รายงานวิจัยฉบับสมบูรณ์

ลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก

ศาสตราจารย์นายแพทย์วรศักดิ์ โชติเลอศักดิ์
หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ภาควิชากุมารเวชศาสตร์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

31 สิงหาคม 2550

รายงานวิจัยฉบับสมบูรณ์

ลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก

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คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

31 สิงหาคม 2550

สัญญาเลขที่ BRG4780017

รายงานวิจัยฉบับสมบูรณ์

ลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก

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หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ภาควิชากุมารเวชศาสตร์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ผู้ร่วมโครงการ

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คุณวุฒิ แพทยศาสตรบัณฑิต

ตำแหน่ง ศาสตราจารย์

สถานที่ทำงาน หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น 11

โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 เขตปทุมวัน กทม. 10330

โทรศัพท์ 02-256-4989, 06-761-2120 โทรสาร 02-256-4911 e-mail: vorasuk.s@chula.ac.th

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ความชำนาญ/ความสนใจพิเศษ Clinical genetics and pediatric endocrinology

สถานที่ติดต่อ หน่วยต่อมไร้ท่อ พันธุกรรม และเมแทบอลิซึม ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น11

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โทรศัพท์ 256-4989 โทรสาร 256-4911

หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การวินิจฉัยและดูแลรักษาทางคลินิกแก่ผู้ป่วย

3. นายแพทย์ สุทธิพงศ์ วัชรสินธุ Suthipong Wacharasindhu, MD

ตำแหน่ง ศาสตราจารย์ คุณวุฒิ แพทยศาสตรบัณฑิต

ความชำนาญ/ความสนใจพิเศษ Clinical genetics and pediatric endocrinology

สถานที่ติดต่อ หน่วยต่อมไร้ท่อ พันธุกรรม และเมแทบอลิซึม ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น11

โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 เขตปทุมวัน กทม. 10330

โทรศัพท์ 256-4989 โทรสาร 256-4911

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หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การดูแลรักษาทางคลินิกแก่ผู้ป่วยกลุ่ม malformation

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หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การดูแลรักษาทางคลินิกแก่ผู้ป่วย

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สถานที่ติดต่อ ฝ่ายศัลยศาสตร์ โรงพยาบาลจุฬาลงกรณ์

ถนนพระราม 4 เขตปทุมวัน กทม. 10330

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ความรับผิดชอบต่อโครงการที่เสนอ ประเมินและดูแลผู้ป่วย

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ถนนพระราม 4 เขตปทุมวัน กทม. 10330

โทรศัพท์ 02-256-4120 โทรสาร 02-256-4120

ความรับผิดชอบต่อโครงการที่เสนอ ประเมินและดูแลผู้ป่วย

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ตำแหน่ง ผู้ช่วยศาสตราจารย์ คุณวุฒิ แพทยศาสตร์บัณฑิต

ความชำนาญ/ความสนใจพิเศษ Neurology

สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น11 โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 เขตปทุมวัน กทม. 10330 โทรศัพท์ 02-256-4951 โทรสาร 256-4911

หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การวินิจฉัยและดูแลรักษาทางคลินิกแก่ผู้ป่วย

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หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การวินิจฉัยและดูแลรักษาทางคลินิกแก่ผู้ป่วย

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ตำแหน่ง ผู้ช่วยศาสตราจารย์ คุณวุฒิ แพทยศาสตร์บัณฑิต

ความชำนาญ/ความสนใจพิเศษ Immunology

สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น11 โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 เขตปทุมวัน กทม. 10330 โทรศัพท์ 02-256-4951 โทรสาร 256-4911

หน้าที่ซึ่งจะต้องปฏิบัติในโครงการวิจัยนี้คือ ให้การวินิจฉัยและดูแลรักษาทางคลินิกแก่ผู้ป่วย

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สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น 9 โรงพยาบาลจุฬาลงกรณ์ ถนนพระราม 4 เขตปทุมวัน กทม. 10330

โทรศัพท์ 02-252-8181 ต่อ 3354 โทรสาร 02-256-4911

ความรับผิดชอบต่อโครงการที่เสนอ ทำการทดลองทางห้องปฏิบัติการ

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ความชำนาญ/ความสนใจพิเศษ Human Molecular Genetics

สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น 9 โรงพยาบาลจุฬาลงกรณ์

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15. นางสาวทิวารัตน์ สินธุวิวัฒน์
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ความชำนาญ/ความสนใจพิเศษ Human Molecular Genetics
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ความชำนาญ/ความสนใจพิเศษ Human Molecular Genetics
สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น 9 โรงพยาบาลจุฬาลงกรณ์
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ความชำนาญ/ความสนใจพิเศษ Human Molecular Genetics
สถานที่ติดต่อ ฝ่ายกุมารเวชศาสตร์ ตึกส.ก. ชั้น 9 โรงพยาบาลจุฬาลงกรณ์
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บทคัดย่อ

รหัสโครงการ BRG4780017

ชื่อโครงการ ลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก

ชื่อนักวิจัย ศาสตราจารย์นายแพทย์วรศักดิ์ โชติเลอศักดิ์

สถาบัน หน่วยเวชพันธุศาสตร์และเมแทบอลิซึม ภาควิชากุมารเวชศาสตร์

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ระยะเวลาโครงการ 1 กันยายน 2547 ถึง 31 สิงหาคม 2550

คณะผู้วิจัยได้ศึกษาลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ ของโรคความพิการแต่กำเนิดและโรคพันธุกรรมเมแทบอลิก ดังนี้ Caffey disease, SATB2 craniofacial mental retardation syndrome, nonsyndromic cleft lip, Tetralogy of Fallot, Bannayan-Riley-Ruvalcaba syndrome, craniofrontonasal syndrome, campomelic dysplasia, Kabuki syndrome, Rickets, Multiple endocrine neoplasia type 1, Conradi-Hunermann-Happle syndrome, Rapp-Hodgkin ectodermal dysplasia syndrome, และ metachromatic leukodystrophy องค์ความรู้ใหม่ที่ได้ ได้รับการตีพิมพ์เป็นบทความทางวิชาการในวารสารวิชาการระดับนานาชาติจำนวน 20 เรื่อง

ความพิการแต่กำเนิดและกลุ่มโรคพันธุกรรมเมแทบอลิกเหล่านี้ เป็นสัดส่วนที่สำคัญของการเจ็บป่วย โดยเฉพาะอย่างยิ่งในเด็ก การหาสาเหตุและเข้าใจการเกิดโรคได้นำไปสู่การวินิจฉัย การรักษาและการป้องกันโรคได้อย่างมีประสิทธิภาพมากขึ้น

คำหลัก: ความพิการแต่กำเนิด โรคพันธุกรรมเมแทบอลิก การกลายพันธุ์ ลักษณะทางคลินิก ความสัมพันธ์ระหว่างการกลายพันธุ์และลักษณะทางคลินิก พยาธิกำเนิดของการกลายพันธุ์ การวินิจฉัยก่อนคลอด

Abstract

Project Code : BRG4780017

Project Title : Clinical, biochemical and molecular features of Thai patients with congenital anomalies or inherited metabolic disorders

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We described clinical manifestation and extended clinical spectrum, studied biochemical features, and identified mutations in patients with dysmorphic syndromes and inherited metabolic disorders including Caffey disease, SATB2 craniofacial mental retardation syndrome, nonsyndromic cleft lip, Tetralogy of Fallot, Bannayan-Riley-Ruvalcaba syndrome, craniofrontonasal syndrome, campomelic dysplasia, Kabuki syndrome, Rickets, Multiple endocrine neoplasia type 1, Conradi-Hunermann-Happle syndrome, Rapp-Hodgkin ectodermal dysplasia syndrome, and metachromatic leukodystrophy. Knowledge gained has been accepted to publish in international journals amounting 20 articles.

These disorders accounted for a significant proportion of morbidity, especially in children. Identification of their etiology and understanding their molecular and biochemical pathogenesis have led to more accurate diagnosis, better treatment and more effective prevention.

Keywords: malformation syndromes, inherited metabolic disorders, mutation analysis, phenotypes, genotype-phenotype correlation, mutation pathogenesis, prenatal diagnosis

เนื้อหางานวิจัย

บทนำ โครงการศึกษาจีโนมมนุษย์ (Human Genome Project) ซึ่งเป็นโครงการทางชีววิทยาที่ใหญ่ที่สุดโครงการหนึ่งในประวัติศาสตร์ของมนุษยชาติ โดยมีจุดประสงค์หลักเพื่อศึกษาการเรียงลำดับ nucleotide ของจีโนมมนุษย์ ได้เสร็จสิ้นลงแล้วเมื่อไม่นานมานี้[1] โรคของมนุษย์เกือบทุกโรคมีสาเหตุมาจากความผิดปกติของสารพันธุกรรมร่วมกับปัจจัยทางสิ่งแวดล้อม เป็นที่ทราบกันอย่างแน่ชัดว่าลักษณะทางคลินิกของโรคที่มีการถ่ายทอดแบบยีนเดียว เช่น thalassemia ขึ้นกับปัจจัยทางพันธุกรรมเป็นอย่างมาก แต่โรคอื่น ๆ เช่น เบาหวาน, ความดันโลหิตสูง, โรคอ้วน, มะเร็ง รวมทั้งลักษณะนิสัยก็อาจมีส่วนได้รับอิทธิพลจากปัจจัยทางพันธุกรรมด้วย การทราบลำดับ nucleotide ในจีโนมมนุษย์ทำให้การค้นพบยีนทั้งที่ก่อโรค (disease causing gene) และที่เพิ่มแนวโน้มการเกิดโรค (susceptibility gene) เร็วขึ้นมาก และการค้นพบยีนที่เกี่ยวข้องกับการเกิดโรคก็มีประโยชน์ตามมาอีกมาก เช่น ทำให้การวินิจฉัยโรคสามารถกระทำได้ตั้งแต่ขณะก่อนมีอาการ ระยะแรกคลอด ขณะตั้งครรภ์ ระยะก่อนการฝังตัว และยังสามารถตรวจพาหะได้อย่างแม่นยำอีกด้วย ตัวอย่างโรคที่ถ่ายทอดทางพันธุกรรมแบบยีนเดียวที่สามารถตรวจได้ด้วยวิธีการทางอณูชีววิทยาในประเทศไทย[2] ได้แก่ achondroplasia[3] pseudoachondroplasia[4] กลุ่มอาการ Crouzon[5] กลุ่มอาการ Apert[6] กลุ่มอาการ Pfeiffer[7] Methylmalonic acidemia[8] กลุ่มอาการ Wiskott-Aldrich[9] Multiple endocrine neoplasia ชนิดที่ 2[10] และกลุ่มอาการ Van der Woude[11] คณะผู้วิจัยจึงได้ถือโอกาสนี้นำความรู้ที่ได้จากโครงการศึกษาจีโนมมนุษย์มาใช้ประโยชน์ในประชากรไทยที่เป็นโรคในกลุ่มความพิการแต่กำเนิด และโรคพันธุกรรมเมแทบอลิก

ความพิการแต่กำเนิด (congenital anomalies) หมายถึง ความผิดปกติทางรูปร่างแต่กำเนิด เป็นกลุ่มโรคที่มีอุบัติการณ์สูง ทารกแรกเกิด 100 คนจะมีผู้ที่มีความพิการแต่กำเนิดชนิดรุนแรงอยู่ 2 ถึง 4 คน[12] โดยมีโรคและกลุ่มอาการที่เป็นสาเหตุของความพิการเหล่านี้เป็นจำนวนมาก ใน website ที่ชื่อ Online Mendelian Inheritance in Man (OMIM) <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM> ซึ่งบันทึกเฉพาะกลุ่มอาการที่เกิดจากยีนเดียวได้บรรจุรายการไว้มากกว่า 10,000 รายการ เมื่อเกิดความพิการแต่กำเนิดขึ้นแล้ว มักเป็นภาระต่อผู้ป่วย ครอบครัวและสังคมเป็นอย่างมาก การให้การวินิจฉัยที่แน่ชัดได้จากลักษณะทางคลินิกและการกลายพันธุ์จะทำให้แพทย์สามารถให้ข้อมูลแก่ผู้ป่วยและครอบครัวได้อย่างถูกต้อง เช่นการพยากรณ์โรคและโอกาสการเกิดซ้ำ ซึ่งเป็นข้อมูลที่มีความสำคัญมากกับครอบครัวในการตัดสินใจต่อไป นอกจากนั้นความพิการแต่กำเนิดบางชนิดยังสามารถป้องกันเพื่อลดโอกาสการเกิดให้น้อยลงได้

โรคพันธุกรรมเมแทบอลิก (inherited metabolic disorders หรือ inborn errors of metabolism) มีความหมายที่ใช้กันโดยทั่วไปคือกลุ่มโรคที่เกิดจากความผิดปกติของกระบวนการย่อยสลาย (catabolism) หรือกระบวนการสังเคราะห์ (anabolism) สารอาหารซึ่งเกิดจากการทำงานของเอ็นไซม์ผิดปกติ การทำงานที่ผิดปกติของเอ็นไซม์จะทำให้เกิดการคั่งของสารตั้งต้นและการขาดของผลิตภัณฑ์ สารตั้งต้นที่คั่งอาจถูกเปลี่ยนไปเป็น metabolites อื่นโดยกระบวนการรอง (minor pathway) ส่งผลให้การทำงานของเซลล์ผิดปกติและเกิดอาการทางคลินิกขึ้น เมื่อเกิดโรคในกลุ่มนี้ ก็เป็นดังเช่นการเกิดความพิการแต่กำเนิด กล่าวคือเป็นโรคที่เป็นภาระต่อผู้ป่วย ครอบครัวและสังคมเป็นอย่างมาก การรักษาให้หายขาดเป็นไปได้ยาก การให้การวินิจฉัยที่แน่ชัดได้จากลักษณะทางคลินิก การตรวจทางชีวเคมีและการกลายพันธุ์จะทำให้แพทย์สามารถให้ข้อมูลแก่ผู้ป่วยและครอบครัวได้อย่างถูกต้อง ซึ่งเป็นข้อมูลที่มีความสำคัญมากกับครอบครัวในการตัดสินใจต่อไป

ผู้ป่วยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิกในแต่ละเชื้อชาติ อาจมีลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ที่แตกต่างกัน เช่น ในคนไทยมีกลุ่มอาการบางอย่างที่ไม่เคยมีรายงานมาก่อนในโลก[13] และโรคหลายโรคก็มีลักษณะทางคลินิก และการกลายพันธุ์ที่แตกต่างจากชนชาติอื่น[4-6, 11, 14, 15] คนญี่ปุ่นที่เป็น Glycogen storage disease type Ia มีลักษณะการกลายพันธุ์ที่เฉพาะในเชื้อชาติญี่ปุ่นเอง กล่าวคือ พบการกลายพันธุ์ชนิด G727T ถึงร้อยละ 88 ของผู้ป่วย[16] ส่งผลต่อการเลือกวิธีการวินิจฉัยให้ได้ความถูกต้องและเสียค่าใช้จ่ายน้อยที่สุด ดังนั้นจึงมีความจำเป็นจะต้องศึกษาลักษณะทางคลินิก ชีวเคมีและการกลายพันธุ์ในคนไทย เพื่อเป็นองค์ความรู้ที่จะช่วยดูแลผู้ป่วยและประชากรไทยให้มีคุณภาพชีวิตที่ดีที่สุด ด้วยค่าใช้จ่ายที่น้อยที่สุด

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วัตถุประสงค์ของโครงการ

เพื่อศึกษาลักษณะทางคลินิก ชีวเคมี และ/หรือลักษณะการกลายพันธุ์ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก

วิธีทดลอง

1. ศึกษาลักษณะทางคลินิกของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก
2. เก็บตัวอย่างต่าง ๆ จากผู้ป่วยและสมาชิกอื่นในครอบครัว เพื่อตรวจทางชีวเคมีและสกัด DNA และ RNA
3. ศึกษาการกลายพันธุ์ของผู้ป่วย และผลของการกลายพันธุ์นั้น ตลอดจนความสัมพันธ์ของการกลายพันธุ์และลักษณะทางคลินิกและชีวเคมี

ผลการทดลอง

คณะผู้วิจัยได้ศึกษาลักษณะทางคลินิก ชีวเคมี และ/หรือลักษณะการกลายพันธุ์ของผู้ป่วยไทยที่มีความพิการแต่กำเนิดหรือเป็นโรคพันธุกรรมเมแทบอลิก สรุปได้ดังนี้

1. คณะผู้วิจัยได้บรรยายหรือเพิ่มลักษณะทางคลินิกของโรคพันธุกรรม ดังนี้
   1.1 Caffey disease (Output ฉบับที่ 1)
   1.2 SATB2 craniofacial mental retardation syndrome (Output ฉบับที่ 2)
2. คณะผู้วิจัยได้พบสาเหตุหรือการกลายพันธุ์ของโรค ตลอดจนกลไกที่การกลายพันธุ์ทำให้เกิดอาการของโรค ดังนี้
   2.1 nonsyndromic cleft lip from mutations in MSX1 (Output ฉบับที่ 3)
   2.2 Tetralogy of Fallot from a de novo derivative chromosome 9 (Output ฉบับที่ 4)

2.3 Bannayan-Riley-Ruvalcaba syndrome from mutations in PTEN (Output ฉบับที่ 5)

2.4 Craniofrontonasal syndrome from mutations in EFNB1 (Output ฉบับที่ 6)

2.5 Campomelic dysplasia from mutations in SOX9 (Output ฉบับที่ 7)

2.6 Kabuki syndrome and BAC array CGH (Output ฉบับที่ 8)

2.7 Cleft lip from mutations in p63 (Output ฉบับที่ 9)

2.8 Rickets from mutations in VDR (Output ฉบับที่ 10)

2.9 Cleft lip from mutations in IRF6 (Output ฉบับที่ 11)

2.10 Multiple endocrine neoplasia type 1 from mutations in RET
(Output ฉบับที่ 12 และ 13)

2.11 Conradi-Hunermann-Happle syndrome from mutations in EBP gene
(Output ฉบับที่ 14)

2.12 Rapp-Hodgkin ectodermal dysplasia syndrome from mutations in p63
(Output ฉบับที่ 15)

2.13 metachromatic leukodystrophy from mutations in ASA (Output ฉบับที่ 16)

3. คณะผู้วิจัยได้นำองค์ความรู้ที่ได้ไปประยุกต์ใช้ในทางคลินิก

3.1 ศึกษาถึงความรู้และพฤติกรรมของหญิงไทยเกี่ยวกับการป้องกันความพิการแต่กำเนิดด้วยกรดโฟลิก เพื่มนำไปเป็นข้อมูลในการเสนอให้หน่วยงานที่เกี่ยวข้องรณรงค์เรื่องดังกล่าว (Output ฉบับที่ 17)

3.2 นำไปทำการตรวจวินิจฉัยก่อนคลอด (Output ฉบับที่ 18 - 20)

สรุป

คณะผู้วิจัยได้บรรยายหรือเพิ่มลักษณะทางคลินิก ได้พบสาเหตุหรือการกลายพันธุ์ของโรค และกลไกที่การกลายพันธุ์ทำให้เกิดอาการของโรคพันธุกรรมหลายโรค ตลอดจนได้นำองค์ความรู้ที่ได้ไปประยุกต์ใช้ในทางคลินิก

OUTPUT

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2. การนำผลงานวิจัยไปใช้ประโยชน์

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

5.1 ผลิตนักวิจัยใหม่ ระดับปริญญาเอก 3 คน ได้แก่

5.1.1 น.ส.สุรัสวดี อัศวรัตน์

5.1.2 น.ส.เพชรรัตน์ เลยกลาง

5.1.3 น.ส.ภัทรา ยี่ทอง

5.2 ผลิตนักวิจัยใหม่ ระดับปริญญาโท 6 คน ได้แก่

5.2.1 นายกรกช พรหมจันทร์

5.2.2 น.ส.สาหร่าย พงศ์จันทนเสถียร

5.2.3 นายประมุข อัมรินทร์นุเคราะห์

5.2.4 น.ส.สาวิตรี รัตนโสภา

5.2.5 น.ส.ณัฐากรณ์ รัตนชาติณรงค์

5.2.6 น.ส.สาธิดา พูนมากสถิตย์

นอกจากนี้งานวิจัยยังเป็นประโยชน์และได้ใช้แล้วกับครอบครัวผู้ป่วยในกลุ่มที่มีความพิการแต่กำเนิดและกลุ่มโรคพันธุกรรมเมแทบอลิกหลายครอบครัว

ภาคผนวก

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Short Report

Expanding the phenotypic spectrum of Caffey disease

Suphapeetiporn K, Tongkobpetch S, Mahayosnond A, Shotelersuk V. Expanding the phenotypic spectrum of Caffey disease. Clin Genet 2007: 71: 280–284. © Blackwell Munksgaard, 2007

Infantile cortical hyperostosis (ICH) is an inherited disorder characterized by hyperirritability, acute inflammation of soft tissues, and massive subperiosteal new bone formation. It typically appears in early infancy and is considered a benign self-limiting disease. We report a three-generation Thai family with ICH, the oldest being a 75-year-old man. A heterozygous mutation for a 3040C→T in exon 41 of *COL1A1* was found in affected individuals, further confirming the autosomal dominance of Caffey disease that is caused by this particular mutation. The novel findings in our studies include short stature and persistent bony deformities in the elderly. The height mean Z-score of the five affected individuals was −1.75, compared to 0.53 of the other seven unaffected individuals giving a p-value of 0.008. Short stature may be partly due to progressive height loss from scoliosis, compression fractures of the spine and genu varus. These features, which have not previously been described, expand the phenotypic spectrum of the Caffey disease.

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Key words: Caffey disease – *COL1A1* – infantile cortical hyperostosis – mutation

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Infantile cortical hyperostosis (ICH) (Caffey disease; OMIM 114000) is characterized by hyperirritability, acute inflammation of soft tissues, and massive subperiosteal formation of the underlying bones typically involving the diaphyses of the long bones, mandible, clavicles, or ribs (1). Its clinical features usually begin before 5 months of age and resolve before 3 years of life (1, 2). It is benign and self-limited. It is inherited as autosomal dominance with incomplete penetrance and variable expressivity (3–5). Even though there are few reports describing the sequelae of the hyperostotic lesions in affected individuals, late recurrence or persistence of symptoms with deformity seems extremely rare (6–8).

A sporadic form of ICH has also been described. In addition, there are several conditions causing cortical bone lesions in children mimicking ICH including prolonged prostaglandin infusion, hypervitaminosis A, and hyperphosphatemia (9–12).

Recently, a novel missense mutation in *COL1A1*, the gene encoding the α1 chain of type I collagen, was found in all affected individuals from three unrelated families (13). All affected individuals were heterozygous for the identical mutation, a 3040C→T transition resulting in the substitution of an arginine by a cysteine at position 836 (R836C), within the helical domain of the α1 chain of type I collagen. Different mutations in *COL1A1* have been found in osteogenesis imperfecta and Ehlers–Danlos syndrome (EDS) (14, 15). Interestingly, some of the clinical features of EDS such as hyperextensible skin and joint hyperlaxity were found in some patients affected with ICH. It was shown that the R134C found in EDS and the R836C found in ICH gave a similar effect on synthesis and function of the collagen fibrils (13). However, the precise

functional link between the R836C mutation and the hyperostotic phenotype seen in ICH is still uncertain and awaits further exploration.

We report a three-generation Thai family with five members affected with ICH. A 3040C→T transition in exon 41 of *COL1A1* was identified. Short stature, persistent bony deformities, and rampant dental caries were present in this molecular proven ICH family.

Material and methods

Clinical subjects

We report a three-generation family with affected members having clinical findings consistent with Caffey disease (see pedigree, Fig. 1, Table 1). Individual I-3 reported to have bow legs since childhood. The deformity persisted and progressed till 75 years of age (Fig. 2a). He had fractures around his left knee twice at the ages of 39 and 43, due to pedestrian struck. His hands were short and stubby. He also had kyphoscoliosis and compression fractures of vertebrae that had not undergone surgery. His radiographs are shown in Fig. 2b–i. II-2 had short, stubby forearms and hands, and bow legs. He had two fractures. The first fracture was on his left forearm that he sustained from playing jumping rope at the age of 13. The second was on his right leg, due to a motorcycle accident, at the age of 19. Although his scoliosis was more severe than that of his father, he did not require surgery (Fig. 2j,k). His radiographs revealed cortical thickening of the affected long bones (data not shown). II-7 was clinically unaffected. However, the radiographs revealed abnormalities of ribs and hands albeit milder severity compared with those of I-3 and II-2 (data not shown). II-11 was noted to have non-painful bowed right leg soon after birth. She easily dislocated and self-reduced her right shoulder. III-3 was a 22-year-old woman who reported to have non-painful curved forearms, and valgal halluces since infancy. III-15 was the proband referred to us at 11 days of age due to swelling and bowing of the right leg (Fig. 2l). She became irritable and cried when passively moving the affected limb. Bone radiographs revealed periosteal elevation and cortical thickening of the diaphyses of the right tibia (Fig. 2m). Other long bones, mandible, clavicles and ribs were unremarkable. Without any medications, swelling and pain on the right leg resolved when she was 2 months old. At 7 months of age, her left leg was swollen and appeared bowed. The radiographs showed her affected left tibia compared with the right (Fig. 2n,o). Again, without any medications, her symptoms gradually subsided and, at 18 months of age, resolved. Some angular deformities of bilateral tibias, however, still persisted (data not shown). The other seven members whose data were available (five were examined by us and two were seen at other medical centers) had normal clinical features, with the mean of the Z score for their height being 0.53 compared to the height mean Z score of −1.75 in the five affected members with the *COL1A1* mutation.

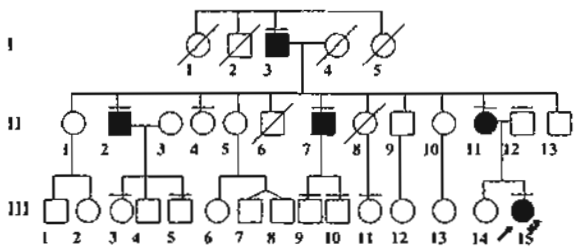

Fig. 1. Pedigree of the family with an autosomal dominant form of Caffey disease. Arrow, proband; blackened symbols, affected individuals; bar above symbol, individuals clinically examined in our center.

Mutation analysis

After informed consent was obtained, genomic DNA was extracted from peripheral leukocytes according to standard protocols. We screened genomic DNA from affected family members and unaffected controls by restriction enzyme digestion of polymerase chain reaction products as previously described (13). One sample from either group with different pattern of restriction enzyme digestion was selected for direct sequencing to further confirm the mutation.

Results

Five affected individuals were heterozygous for a 3040C→T transition in exon 41 of *COL1A1* (Table 1, Fig. 3). Surprisingly, patient III-3 who had clinical features consistent with ICH did not have the mutation as shown by the restriction enzyme digestion (Fig. 3) and direct sequencing (data not shown). The mutation analyses of this individual were confirmed by repeated studies using DNA from two separate blood collections.

Discussion

We described a Thai family with the proband presented with clinical and radiographic features

Table 1. Clinical, radiological and molecular findings of affected members

Features	I-3	II-2	II-7	II-11	III-15
Sex	M	M	M	F	F
Age (years)	75	50	44	30	1
Height (cm)	147	150	156.7	150.6	71.5
Z-score	−3.20	−2.68	−1.39	−0.61	−0.88
Head circumference (cm)	56.5	58	59.5	55.5	45
Onset of cortical hyperostosis	NA	NA	NA	Soon after birth	Soon after birth
Angular deformity	Both forearms and legs	Both forearms and legs	N	Right leg	Both legs
History of fractures	Twice	Twice	N	N	N
Blue sclerae	N	N	N	N	N
Rampant dental caries	Y	Y	Y	Y	N
Hyperextensible skin	N	N	N	N	N
Joint hyperlaxity	Y	N	N	Y	N
Scoliosis	Y	Y	N	N	N
3040C→T (R836C)	Y	Y	Y	Y	Y

M, male; F, female, Y, yes; N, no, NA, not applicable.

consistent with ICH. There were multiple affected members showing an autosomal dominant condition.

We had an opportunity to examine 11 members of this family. Five were found to have the *COL1A1* 3040C→T mutations. Additional striking phenotype that we observed in this family was short stature. Short stature was not previously found to be part of the disorder (13). Comparing height of these five individuals with that of the other seven clinically unaffected, we found that the means of the Z scores for their heights were statistically different ($p = 0.008$). We did not include III-3, who had curved forearms but did not have the *COL1A1* 3040C→T mutations in her leukocytes, in either group. Short stature could partly be caused by loss of adult height from kyphoscoliosis, compression fractures of vertebrae, and lower limb deformities as seen in our oldest 75-year-old man and his affected first son.

ICH has been considered a benign and self-limited disorder. Our 75-year-old patient, the oldest individual ever reported with ICH, and his 50-year-old affected first son have been generally healthy despite persistent deformity of the long bones. Even though persistent deformities of the affected bones have been previously noted (6–8), this study described the first molecular proven Caffey family with persistent deformities. Based on these data, we propose that short stature and persistent bony deformity should be included in the clinical spectrum of Caffey disease.

Rampant dental caries were observed in all mutation positive members, except III-15 who was still very young. However, the dental problems were also observed in many other family members without the mutation. Therefore, whether this phenotype is part of ICH needs further studies.

A heterozygous mutation for a 3040C→T in a CpG dinucleotide of exon 41 of *COL1A1* was identified. This similar mutation was previously described in a study of three unrelated kindreds from Australia and Canada with an autosomal dominant form of ICH (13). Our study in a large Thai family further confirmed that familial Caffey cases are caused by this particular mutation. The fact that all the familial cases studied so far had the same mutation, regardless of their ethnicities, supports the previous observation that the 3040C→T transitions are recurrent, making it a mutational hot spot in *COL1A1*.

In this family, we found two members with discrepancy between genotype and phenotype. II-7 had some minor radiographic findings without clinical features of Caffey disease but had the mutation. Incomplete penetrance and variable expressivity have been previously observed in Caffey disease (3–5). It has been shown that 21% of the individuals carrying the R836C substitution do not develop the disease (13). More interestingly, the heterozygous *COL1A1* 3040C→T mutation was not found in a 22-year-old woman with curving deformity of bilateral forearms since early childhood. Radiographs taken during her first visit at 22 years of age revealed cortical thickening of the affected bones. Unexpectedly, the 3040C→T mutation was not detected in her genomic DNA extracted from the peripheral leukocytes in two separated DNA samples obtained from blood drawn on two occasions. One possibility is that she is a mosaic for the inherited mutation. The occurrence of the nucleotide substitution in an already

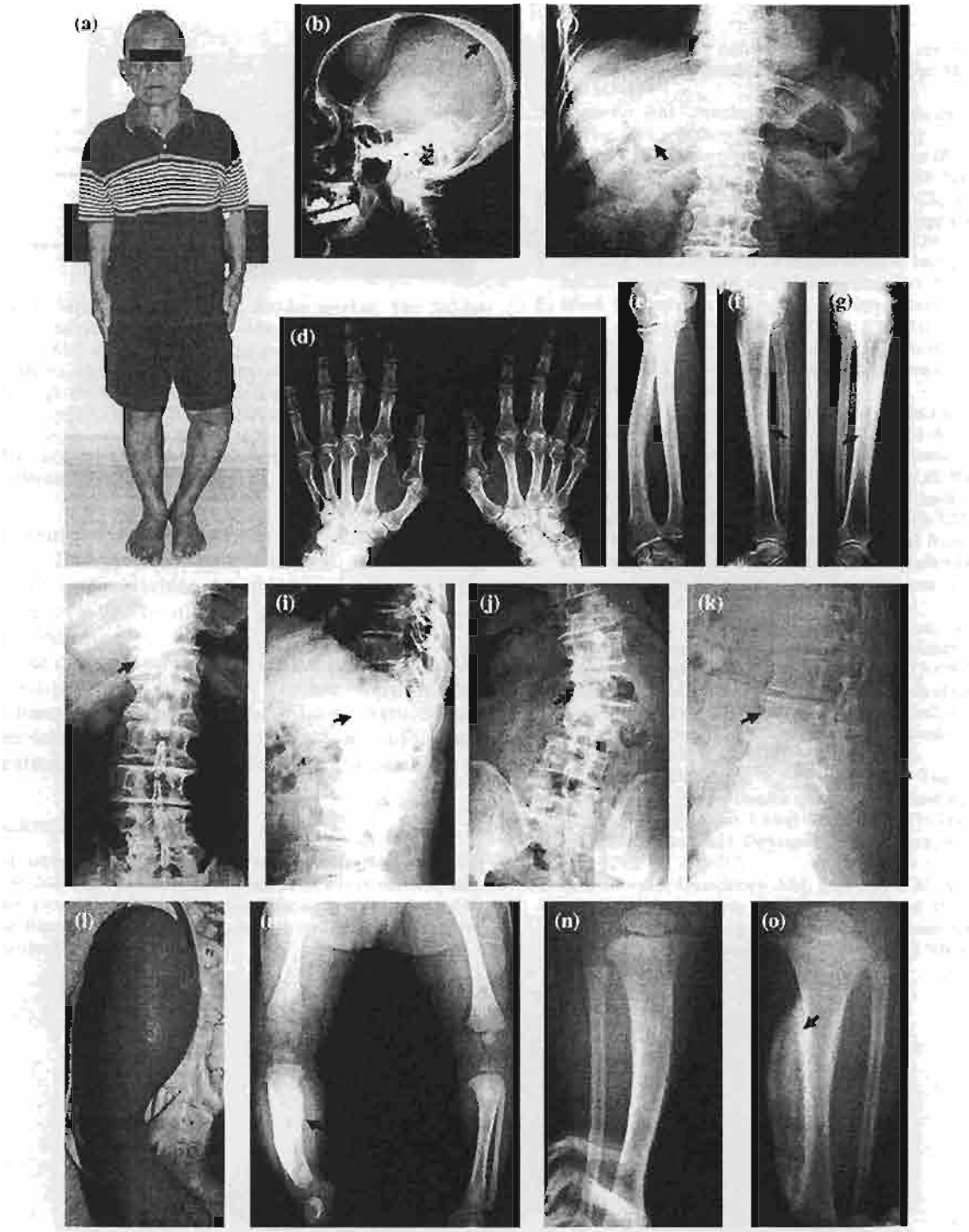

Fig. 2. Clinical and radiological features of affected individuals. (**a**) Photograph of the oldest individual (I-3) with Caffey disease. Radiographs of patients I-3 (**b–i**), II-2 (**j, k**), and the proband, III-15 (**l–o**) showing (**b**) cortical thickening of the cranial vault (**c**) ribs with paddle-like shape (**d**) hands with short metacarpals (**e**) angular deformity with cortical thickening of the radius (**f, g**) anterior bowing with cortical thickening of the right and left tibias respectively (**h, i**) kyphoscoliosis with compression fracture of T12 (**j, k**) scoliosis with spondylosis of L2. Photograph of the proband (**l**) showing swelling of the right leg, which is matched to the radiograph (**m**) showing periosteal elevation and cortical thickening of the diaphyses of the right tibia. Radiographs of bilateral legs showing (**n, o**) cortical hyperostosis with anterior curvature of the right tibia and periosteal elevation and cortical thickening of the left tibia, respectively. Arrows indicated the affected bones.

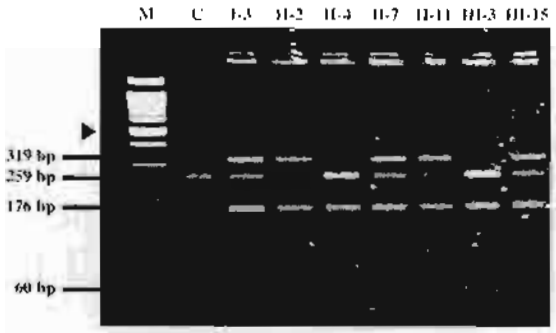

Fig. 3. Mutation analysis. M: 100-bp marker. The 500-bp band indicated by an arrow head. C: unaffected control. Restriction enzyme analysis of polymerase chain reaction products showing the mutant allele lacking one of the cleavage sites for the restriction endonuclease *HpyCH4IV* resulting in bands of 319 and 176 bp and the wild-type allele with bands of 259, 176 and 60 bp. Patients I-3, II-2, II-7, II-11 and III-15 were heterozygous for C→T mutation at nucleotide 3040 of *COL1A1*.

mutant nucleotide has been previously described (16). DNAs from other tissues are, unfortunately, unavailable. Another possibility is that she is affected by another disorder, not linked to the mutation.

The clinical and molecular characteristics of the inherited form of Caffey disease were further delineated in our study. Short stature and persistence of bony deformity were additional features found in Thai affected individuals.

Acknowledgements

We are grateful to all the family members for their invaluable contributions. We also wish to thank Dr Pairoj Chotivitayatarakorn for referring patients to us. This study was supported by the Research Unit Fund, Chulalongkorn University, and the Thailand Research Fund.

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HUMAN MUTATION 28(7), 732–738, 2007

RESEARCH ARTICLE

Heterozygous Nonsense Mutation *SATB2* Associated With Cleft Palate, Osteoporosis, and Cognitive Defects

Petcharat Leoyklang,[1] Kanya Suphapeetiporn,[1] Pichit Siriwan,[2] Tayard Desudchit,[1] Pattraporn Chaowanapanja,[3] William A Gahl,[4] and Vorasuk Shotelersuk[1]*

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Communicated by Iain McIntosh

Studies of human chromosomal aberrations and knockout (KO) mice have suggested *SATB2* as a candidate gene for a human malformation syndrome of craniofacial patterning and brain development. Of 59 unrelated patients with craniofacial dysmorphism, with or without mental retardation, one 36-year-old man had a nonsynonymous mutation in *SATB2*. The affected individual exhibited craniofacial dysmorphisms including cleft palate, generalized osteoporosis, profound mental retardation, epilepsy and a jovial personality. He carries a de novo germline nonsense mutation (c.715C>T, p.R239X) in the exon 6 of *SATB2*. Expression studies showed that the mutant RNA was stable, expected to produce a truncated protein predicted to retain its dimerization domain and exert a dominant negative effect. This new syndrome is the first determined to result from mutation of a gene within the family that encodes nuclear matrix-attachment region (MAR) proteins. Hum Mutat 28(7), 732–738, 2007. Published 2007 Wiley-Liss, Inc.[†]

KEY WORDS: *SATB2*; cleft palate; osteoporosis; cognitive deficit; epilepsy

INTRODUCTION

Cleft palate has various known and suspected genetic etiologies in humans [Stanier and Moore, 2004; Park et al., 2006]. One possibly causative gene is *SATB2*, encoding a cell type–specific transcription factor that functions as a regulator of the transcription of large chromatin domains. Unlike classic transcription factors that bind individual target genes to regulate transcription, SATB2 binds to multiple sites, influencing chromatin organization and structure and orchestrating the transcription of several genes. SATB2 is a target for SUMOylation, a reversible protein modification that modulates its activity as a transcription factor [Dobreva et al., 2003]. The *SATB2* (MIM# 608148) gene resides on chromosome 2q32–q33, spans 191 kb, and contains 11 exons. Its open reading frame begins in exon 2, with the first stop codon in exon 11, predicting a 733–amino acid protein. The protein contains a Pfam-B_10016 domain required for dimerization (residues 57–231), two CUT domains (352–437 and 482–560), and a homeodomain (614–677) [FitzPatrick et al., 2003].

Diverse evidence links SATB2 to the occurrence of cleft palate. Rodent studies indicate that Satb2 plays an important role in craniofacial patterning [Britanova et al., 2006b; Dobreva et al., 2006], and brain development [Britanova et al., 2005, 2006a; Szemes et al., 2006]. Two patients with isolated cleft palate and balanced chromosomal translocations had disruptions in *SATB2* [FitzPatrick et al., 2003]. Four other patients exhibited the combination of cleft or high palate and interstitial deletions at 2q32–q33 [Van Buggenhout et al., 2005]. In Singaporean and Taiwanese patients, SNPs in the *SATB2* gene have been found in significant association with isolated cleft lip and palate [Beaty et al., 2006]. Despite these findings, mutation analysis has failed to identify a definitive intragenic *SATB2* mutation in any individual with an isolated oral cleft [FitzPatrick et al., 2003; Vieira et al., 2005]. Consequently, we examined the *SATB2* gene in 59 individuals with craniofacial dysmorphism with or without mental retardation. One member of this group, with craniofacial dysmorphism and profound mental retardation, carries a de novo nonsense mutation in *SATB2*.

PATIENTS AND METHODS

Patients

A total of 59 unrelated patients were studied under the auspices of the Thai Red Cross, a national charity organization devoted to providing clinical care for the poor. Subjects were recruited between 1999 and 2006 from 15 medical centers throughout Thailand. The study was approved by the local Ethics Committee.

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written informed consent was obtained from each person included in the study.

Clinical Studies

For the proband, a CT scan of facial bones was performed with Somatom sensation 16 scanner (Siemens, Forchheim, Germany). The images were obtained using a soft tissue and bone algorithm with 0.75-mm thin collimation, spiral technique. The scan covers the vertex to the inferior aspect of the mandible. Axial, coronal, sagittal, and three-dimensional (3D) images were reconstructed. An MRI of the brain was performed using GE Signa Excite 1.5 Tesla (GE, Fairfield, CT). The imaging protocols were spin echo spin lattice relaxation time–weighted imaging (SE T1WI), fast spin echo spin spin relaxation time–weighted imaging (FSE T2WI), gradient recalled echo spin-spin interaction time plus magnetic field inhomogeneities and susceptibility effects–weighted imaging (GRE $T2^*$WI), fluid attenuation inversion recovery (FLAIR) imaging, echo planar imaging (EPI) diffusion-weighted imaging, and post-contrast SE T1WI with intravenous injection of gadolinium 0.1 mmol/kg. An overnight video-electroencephalography (EEG) was performed on a 32-channel digital video-EEG machine (Stellate, Montreal, Quebec, Canada) with spike detector. Total duration of the study was 12 hr.

Mutation Analysis

Genomic DNA was isolated from peripheral blood, obtained at the time of blood typing and hematocrit determination, according to established protocols. Intronic primers were used to amplify fragments encompassing exons 2–11 of the *SATB2* gene (Table 1). PCR reactions were carried out in a 20 μl volume containing 50 ng genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.5 unit Taq polymerase, using the following parameters: 30 s at 94°C, 30 s at the annealing temperature (Table 1), and 30 s at 72°C for 35 cycles. PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendations, and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). Analyses were performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). When the results indicated a possible new variant, the sample was resequenced. The position of mutations corresponds to the coding sequence for *SATB2* (RefSeq NM_015265.1), with +1 corresponding to the A of the ATG translation initiation codon.

Restriction Enzyme Analysis

The nonsynonymous coding variant was verified by restriction enzyme digestion of the patient's PCR products. *SATB2* exon 6 was PCR-amplified using a mutagenic forward primer to incorporate a *Bss*SI recognition site, 5′ TAATTATCACTTT TATCTCTT AACAGTGGAAAGAGTGGCA 3′, and a reverse primer, 5′-TGTCCTGAGATCCATAAAGC-3′. The PCR conditions were 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C for 35 cycles. The PCR products were digested with *Bss*SI according to the manufacturer's specifications (New England Biolabs, Ipswich, MA) and electrophoresed on a 3% agarose gel stained with ethidium bromide.

The proband's parents were examined for the variant by sequencing and restriction enzyme analysis. Paternity and maternity were confirmed by typing 15 microsatellite markers on 13 different chromosomes (data not shown). Thai control individuals (n = 105) were analyzed by restriction enzyme analysis.

RNA Studies

Total RNA was isolated from white blood cells using a QIAamp® RNA blood mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using ImProm-II™ reverse transcriptase (Promega, Madison, WI), according to the company's recommendations. PCR amplification of the *SATB2* cDNA partial exons 5 and 6 was performed using primers cDNA-F4, 5′-CCAATGTGTCAGCAACCAAG-3′ and cDNA-R1, 5′-TGGT GAATTTGGCTGTGAGG-3′. We used 2 μL of first-strand cDNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, and 0.5 U Taq DNA polymerase in a total volume of 20 μL. The PCR conditions were 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C for 26, 28, 30, 32, 35, 37, 40, or 42 cycles. The 199-bp PCR products were treated with ExoSAP-IT and subjected to direct sequencing.

RESULTS

Case Report

The patient is a 36-year-old man born at term by vaginal delivery to a 24-year-old, gravida 3, para 2 mother and a 32-year-old unrelated father. The pregnancy and labor were uncomplicated. Cleft palate was apparent in infancy, but was not surgically corrected. The patient walked at 4 years of age and could speak a single word, "nee," meaning mother, from 6 years of age. He had several febrile seizures during childhood and developed generalized tonic-clonic seizures at age 26 years. His mother reported that he had frequent and severe respiratory tract infections during childhood, worse than those of his three unaffected siblings. At age 35, while walking, the patient fell and fractured his left tibia, left fibula, and right third metatarsus. He required assistance for daily routines including dressing, washing, and eating. His mood was jovial. Physical examination at age 36 years revealed a height of

TABLE 1. Oligonucleotides and PCR Condition for *SATB2* Mutation Analysis

Exon	Primer sequences for PCR (5′ to 3′) Forward	Reverse	Annealing temperature (°C)	Product size (bp)
2	GTCCCTGTGCGTTTTATTGC	GCAACCTGGAATTCACTTCC	57	350
3	TGTTGCTTCCCTTCTCATCG	TACTGCTCACTAGGAAATGC	57	390
4	AATATCTGAGTGGGCCTTGG	CAGGACATGCTGATCTTTGC	59	330
5	AGCATTTCTTCTGAAGCTCC	ACAGTTGTTTAAGCAGAAGG	55	325
6	TAGTGCAGGTTATAAAGTGC	TTTTAAGGGAGCCAACTAGG	55	352
7	ACTTTTATGCTGGAGCTTCC	TGTCCTGAGATCCATAAAGC	55	725
8	TGTGAGGTTCTTGACATCAC	GTCTCTCAATGTTTGAGGGA	55	431
9	GCATCAGCTGACTGAAATCC	GAACATGACAGGTTTCTTGG	57	379
10	ATGTACTGTGATGGCACTGG	GAAGTTGGTGTGGTGTGTGC	59	383
11	AATGACTATAGCTTACCTCC	TGAAAGCAGAAAATCCTTGG	55	632

165 cm (25th centile), weight 40 kg (less than third centile), and head circumference 56 cm (50th centile). He had gum hyperplasia, slight micrognathia, and deviation of the chin to the right (Fig. 1A and B). His chest, abdomen, genitalia, and extremities were unremarkable.

Blood counts, urinalysis, blood urea nitrogen (BUN), creatinine (Cr), electrolytes, chromosomal analysis, immunoglobulin (Ig) G, IgM, IgA, and IgE were all within normal ranges. Plain radiographs of his facial bones, chest, hands, legs, feet, spine, and urinary system showed generalized osteoporosis, narrow bilateral carpometacarpal and tarsometatarsal joints (Fig. 1I), loss of normal kypholordotic curvature of the thoracolumbar spine (not shown), and anterior bowing of the tibias (Fig. 1J). Old healed fracture lines were observed at the proximal left fibular shaft, the distal left tibial shaft, and the lateral side of the midright third metatarsal shaft.

A CT scan of the facial bones revealed bilateral asymmetric mandibular hypoplasia (Fig. 1C), wide mandibular angles, anterior overbite of the upper teeth with marked anterior-pointing incisors (Fig. 1D), a midline cleft palate, small sizes and abnormal shapes of the bilateral maxillary sinuses (Fig. 1E), and mild ocular hypertelorism with the anterior and midinterorbital distances measuring 30.9 mm (normal range: 19.9–27.7 mm) and 34.0 mm (normal range: 22.3–32.7 mm), respectively (Fig. 1F). [Waitzman et al., 1992] In addition, slightly short zygomatic arches were observed, and the mandibular condylar heads were flattened rather than oval (not shown). A brain MRI showed no demonstrable intracranial abnormality (Fig. 1G). However, cervical spine imaging revealed a hyperintense mass on T2-weighted image at the right side of C3-4 vertebrae (Fig. 1H), considered an incidental finding.

An awake EEG revealed a background of bilateral symmetrical 8 to 9 Hz, 50 to 75 μV waves, attenuated by eye opening. The sleep EEG revealed a mix of generalized, irregular, slow 2 to 3 Hz, 25 to 50 μV waves and 4 to 5 Hz, 25 to 50 μV waves. Bilateral synchronous sleep spindles, 12 to 13 Hz, 50 to 75 μV waves and vertex transient, 75 to 100 μV waves, were noted in the paracentral head areas. Multiregional sharp waves phase reversing at T3, T5, T4, T6, C3, and C4 every 2 to 5 minutes were found (Fig. 2). Occasionally, these sharp waves spread and lateralized to the right and left hemispheres. Rare short runs of repetitive spikes were seen. No clinical or EEG seizures occurred during the period of recording.

Molecular Analyses

Of 59 patients investigated with craniofacial dysmorphism, one exhibited an alteration in the coding region of *SATB2*. The mutation was a c.715C>T transition in exon 6 (Fig. 3A), converting an arginine (CGA) into a premature TGA stop codon (p.R239X). Each parent had only the wild-type allele, indicating that the patient's c.715C>T mutation occurred de novo. No other sequence variants were found in the patient's *SATB2* coding regions. Leukocyte mRNA of the *SATB2* gene, quantitated by reverse transcription and PCR sequencing after 32 cycles of PCR,

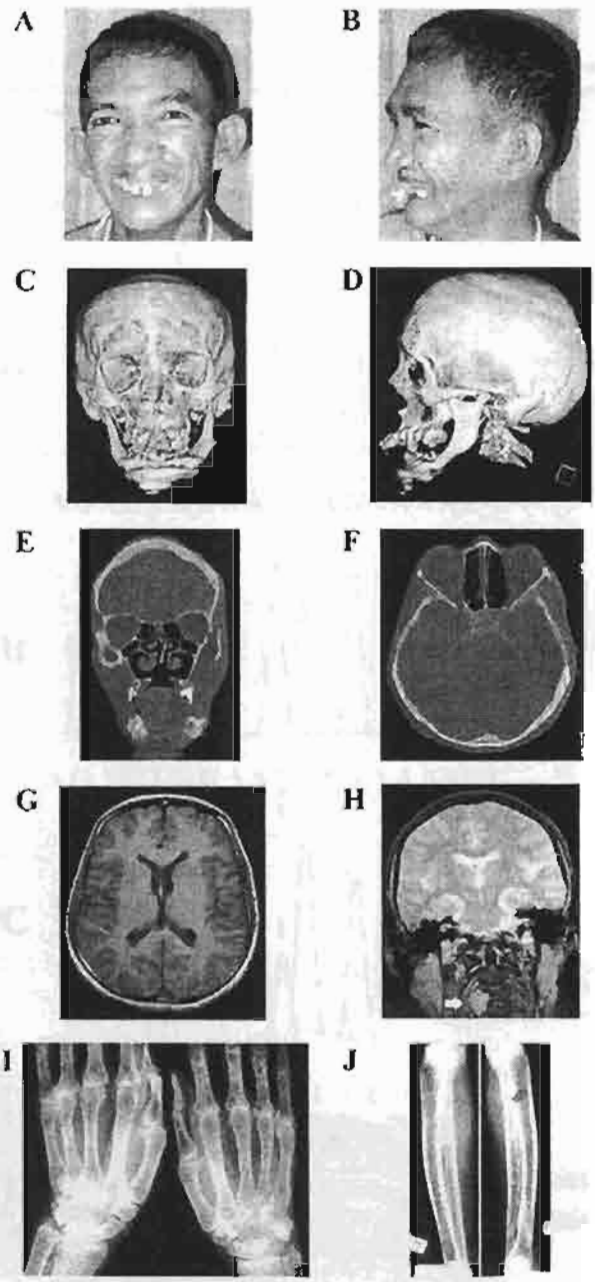
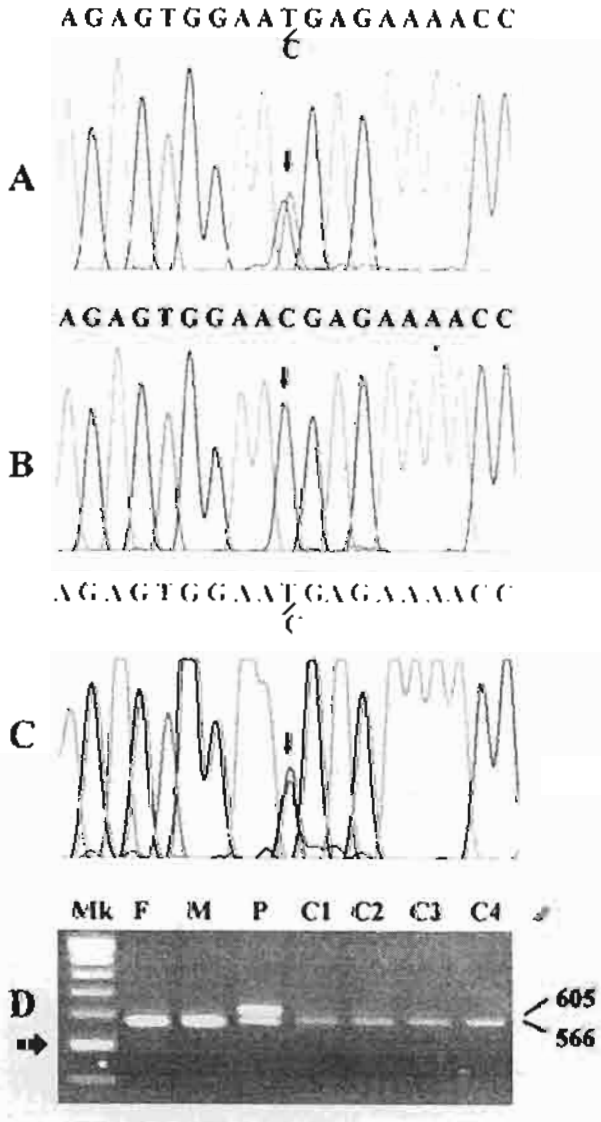


FIGURE 1. **Clinical features and imaging studies of the patient with a *SATB2* nonsense mutation. A,B:** Craniofacial features of the patient, anterior and lateral views, respectively. **C,D:** Reconstructed images of the CT scan of facial bones, anterior and lateral views, respectively. Note the anterior open bite with marked anterior-pointing incisors. Shallow mandibular notches and wide mandibular angles reflect mandibular hypoplasia, which is more severe on the right resulting in right-sided pointed chin. Motion artifacts are seen at the mid-body of mandible causing irregular blur image. **E:** The coronal plane, bone window of the CT scan of facial bones. A 2-cm wide, midline cleft palate and absent vomer are seen. Note the small size and abnormal shape of the bilateral maxillary sinuses, high position of the inferior maxillary walls and wide inferior meati bilaterally. **F:** The axial plane, bone window of the CT scan of facial bones demonstrating mild hypertelorism. **G:** An axial T1WI of the MRI of the brain with no demonstrable abnormalities of the brain parenchyma. **H:** A T2WI of the MRI of the brain demonstrating a 2.3 × 1.9 cm hyperintense mass at the right side of C3-4 vertebrae (arrow). **I,J:** Plain radiographs of the wrists in anteroposterior view and legs in lateral view demonstrating narrowing of carpometacarpal joints and anterior bowing of both tibias, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 2 A composite picture of the electroencephalogram demonstrates multiregional sharp waves phase reversing at T3, T5, T4, T6, C3, and C4, with sharp waves lateralized to the left hemisphere. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



while in the exponential phase (data not shown), was present in equivalent amounts for the mutant and wild-type alleles (Fig. 3C).

The c.715C>T transition was confirmed by digestion of the PCR products using a mutagenic primer with the restriction enzyme BssSI, whose recognition site was removed by the point mutation (Fig. 3D). The mutation was not previously reported and was not detected in 105 control individuals (210 alleles).

DISCUSSION

Studies of human chromosomal aberrations and knockout (KO) mice have suggested *SATB2* as a candidate gene for human malformation syndromes involving abnormalities in craniofacial patterning and brain development. This led us to investigate *SATB2* in individuals with craniofacial malformations with or without mental retardation. We identified the first human individual having a pathogenic SATB2 point mutation. He had craniofacial dysmorphism, generalized osteoporosis, profound mental retardation, epilepsy, and a pleasant personality.

Our patient's clinical features are similar but not identical to those of model mice and other syndromic patients (Table 2). His craniofacial dysmorphisms, including maxillary malformation, mandibular hypoplasia, and cleft palate, resembled those of mice lacking *Satb2* [Dobreva et al., 2006]. The asymmetric mandibular hypoplasia in our patient is consistent with the asymmetric snout of adult heterozygous *Satb2*$^{+/-}$ mice [Britanova et al., 2006b]. The

FIGURE 3. *SATB2* gene mutation. **A,B:** DNA sequence analyses of *SATB2* from the patient and his father, respectively. The arrows indicate the heterozygous mutation, T/C, in the patient and the wild-type only, C, in the father. **C:** The patient's leukocyte RNA sequence analysis. The arrow indicates the mutant base, T, is present in an amount equal to that of the wild-type base, C. **D:** Restriction enzyme analysis. With a mutagenic primer, the mutant allele in the patient (P) eliminates the *Bss*SI recognition site resulting in an uncut 605-bp band, presenting along with a 566-bp band of the wild-type allele. The 39-bp band of the cut wild-type allele is not visible. The patient's father (F), mother (M), and controls (C) have only the wild-type allele. The 500-bp band of the 100-bp marker (M) is indicated by an arrow. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 2. Characteristics of Our Patient Compared With Those of *SATB2* KO Mice, Patients With Chromosomal Abnormalities Involving *SATB2*, and Patients With Known Syndromes Including Cleft Palate, Osteoporosis, and Mental Retardation

	Our patient	*SATB2* KO mice [Britanova et al., 2006b; Dobreva et al., 2006]	Patients with chromosomal translocation interrupting *SATB2* [Brewer et al., 1999]	del(2)(q32.2q33) deletion syndrome [Van Buggenhout et al., 2005]	Snyder-Robinson syndrome (MIM# 309583)	Larsen syndrome (MIM# 245600)	Lowry-Maclean syndrome (MIM# 600252)	Rothmund-Thomson syndrome (MIM# 268400)
Microcephaly	A	P	A	P	A	A	P	A
Cleft palate	P	P	P	P	P	P	P	P
Other craniofacial dysmorphism	Hypertelorism, short zygomatic arches, anterior overbite, gum hyperplasia, asymmetric mandibular hypoplasia, wide mandibular angle	Truncation of mandible, shortening of nasal and maxillary bones, missing incisor teeth, small tongue	Long narrow face, hypotelorism, squint, prominent nasal bridge, underhanging columella, small mouth, micrognathia	Thin hair, high nasal bridge, micrognathia, macroglossia, maxilla hypoplasia	Facial asymmetry	Small face, prominent forehead, hypertelorism, glaucoma, depressed nasal bridge	Craniosynostosis, glaucoma, delayed dentition, beaked nose	Alopecia, frontal bossing, prognathism, cataracts, microphthalmia, small nose, microdontia
Stature	Normal	NA	Normal	Short	Tall	Short	Short	Short
Mental retardation	Profound	NA	Mild delayed development	Severe	Mild to moderate	P	P	P
Epilepsy	P	NA	A	P	P	A	A	A
Osteoporosis	P	P	NA	NA	P	P	P	P
Personality-behavior	Happy	NA	NA	Happy, aggression, anxiety	NA	NA	NA	NA
Others	Narrow carpometacarpal and tarsometarsal joints	Died immediately after birth	Long and slender fingers	Inguinal hernia, wide-based gait, sleep problems, self-mutilation	Hypotonia, unsteady gait, marfanoid habitus, kyphoscoliosis, cryptorchidism	Dislocation of joints, club feet, scoliosis, skin laxity, hypotonia	Eventration of diaphragm, congenital heart defect	Forearm reduction, small hands and feet, poikiloderma, photosensitivity, ectodermal dysplasia
Mutated gene	*SATB2*	*Satb2*	*SATB2*	NA	*SMS*	Unknown	Unknown	*RECQL4*
Chromosome	2q32	NA	2q32	2q32-q33	Xp22	Unknown	Unknown	8q24

A, absent; P, present; NA, not applicable or not available.

abnormal craniofacial patterning in *Satb2* KO mice involves desuppression of the *Hoxa2* expression. Hoxa2 inhibits bone formation, and Satb2 suppresses *Hoxa2* expression, so the absence of Satb2 inhibits osteoblast differentiation [Ellies and Krumlauf, 2006]. Craniofacial development is sensitive to *Satb2* dosage, with full functional loss resulting in amplification of the defects seen in Satb2 haploinsufficiency [Britanova et al., 2006b]. Our patient's craniofacial abnormalities are more severe than those associated with the *SATB2* haploinsufficiency of 2q32–q33 deletions and translocations, perhaps due to the possible dominant negative nature of his mutation. In addition to abnormal skeletal patterning, generalized osteoporosis and fractures occurred in our patient, consistent with the proposed role of SATB2 in regulating skeletal development and osteoblast differentiation [Dobreva et al., 2006].

Our patient also has profound mental retardation and epilepsy. Satb2 is expressed in mouse and rat developing neocortex and is involved in the control of neuronal differentiation and migration [Britanova et al., 2005, 2006a; Szemes et al., 2006]. Our patient has a normal head circumference, whereas the *Satb2*$^{+/-}$ mice were microcephalic, with increased microcephaly in *Satb2*$^{-/-}$ [Britanova et al., 2006b]. The two patients with translocations involving *SATB2* haploinsufficiency had mild learning disability in addition to cleft palate [FitzPatrick et al., 2003]. Our patient's pleasant personality resembles that of patients with the del(2)(q32.2q33) interstitial deletion syndrome [Van Buggenhout et al., 2005].

SATB2 regulates expression of the immunoglobulin mu gene [Dobreva et al., 2003], and the patient had frequent respiratory tract infections, but his immunoglobulin levels, including IgM, were normal. The hyperintense mass at the right side of our patient's C3-4 vertebrae was thought to be a schwannoma, mesenchymal tumor, or an expansile benign bony tumor; the relationship to *SATB2* mutations requires further investigation. Since chromosomal aberrations affect many different genes, the contribution of *SATB2* mutations to the phenotypes of patients with chromosomal translocations and interstitial deletions is not known. A T190A mutation in *SATB2* has recently been identified in a Filipino patient with isolated bilateral cleft lip and cleft palate. Although not found in 186 matched controls and in a panel of 1,064 Centre d'Etude du Polymorphisme Humain (CEPH) controls, this mutation involves a base that is not highly conserved across species, is predicted to be benign by PolyPhen (www.bork.embl-heidelberg.de/PolyPhen; Peer Bork EMBL, Heidelberg, Germany), and was present in his unaffected mother. The presence of only cleft palate and not cleft lip in our patient, in individuals with chromosomal aberrations involving *SATB2*, and in *Satb2* KO mice makes the etiologic role of the *SATB2* T190A mutation further suspect. In contrast, the pathogenic role of our patient's c.715C>T mutation in *SATB2* is supported by three lines of evidence. First, this nonsense mutation is expected to result in a truncated protein of 238 residues, instead of 733 residues, lacking the two functional CUT domains and homeodomain. Second, his molecularly-confirmed biological parents had only wild-type alleles, indicating that the mutation is de novo. Third, the mutation was absent from 105 ethnically-matched control individuals. Therefore, the c.715C>T mutation is the first definitive intragenic mutation of *SATB2* in a human; a previous of 70 unrelated isolated cleft palate patients failed to identify any pathogenic *SATB2* mutations [FitzPatrick et al., 2003].

The presence of the mutant RNA in our patient's leukocytes indicated lack of nonsense-mediated mRNA decay. Rather, translation is predicted to produce a truncated protein of 238 residues retaining its dimerization domain (residues 57–231). Nevertheless, impaired function could result in haploinsufficiency. With respect to the mechanism of the defect involved, it should be noted that the transcripts used in our study were from adult leukocytes, while *SATB2* acts early in development; the transcript levels should be interpreted with caution. As more patients with *SATB2* mutations are recognized, the syndrome will be better defined. Patients with phenotypes similar to that of our patient (Table 2), but with an unidentified causative gene, should be considered candidates for *SATB2* mutation analysis.

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ORIGINAL ARTICLE

MSX1 mutations contribute to nonsyndromic cleft lip in a Thai population

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Abstract Previous studies observed that *MSX1* mutations could contribute to nonsyndromic cleft lip with or without cleft palate (CL/P) in some populations. Of the proposed pathogenic mutations, the P147Q variant was predominant in Vietnamese and present in Filipino populations. We investigated whether *MSX1* mutations also contribute to nonsyndromic CL/P in the Thai population. Specifically, we performed mutation analysis covering all the coding regions of the *MSX1* gene for 100 Thai patients with nonsyndromic CL/P. A total of eight variant sites were identified. Six were in coding regions, including four nonsynonymous changes, 101C > G (A34G), 440C > A (P147Q), 799G > T (G267C), and 832C > T (P278S). The G267C and P278S variants were predicted to be "probably damaging" by PolyPhen, changed themselves as potential exonic splicing enhancers for serine/arginine-rich proteins, and were not present in 162 control individuals of Thai ethnic background. Unlike all of the previously reported potential missense mutations in *MSX1*, these two novel potential mutations were found in exon 2 on the C-terminal side of the homeodomain protein. Moreover, in contrast to previous reports, we found the P147Q variant in 8 out of 100 Thai controls and an association between the variant and CL/P in our population could not be detected, suggesting that it is not pathogenic. Our data support that *MSX1* mutations are found in 2% of cases of CL/P and should be considered for genetic counseling implications, but suggest that the P147Q variant is not pathogenic.

Keywords Nonsyndromic cleft lip · *MSX1* · Mutations · Association · Haplotype

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Introduction

Nonsyndromic cleft lip with or without cleft palate (CL/P) is the most common craniofacial anomaly. It has a prevalence of approximately 1 in 600 among Thai newborns (Shotelersuk et al. 2003). Environmental and genetic factors have been implicated in CL/P and several different loci and genes have been associated with them (Jugessur and Murray 2005).

In 1994, *MSX1* first emerged as a candidate based on the CL/P and foreshortened maxilla phenotype in the knockout mouse (Satokata and Maas 1994). Several association studies of the gene with CL/P and cleft palate only (CPO) further supported the role of *MSX1* in nonsyndromic clefting in different populations (Lidral et al. 1998; Blanco et al. 2001; Jugessur et al. 2003). Later on, a study of a Dutch family with tooth agenesis and various combinations of CL/P and CPO showed a nonsense mutation in *MSX1*, suggesting that

disease-causing mutations in *MSX1* might be etiological in a portion on nonsyndromic CL/P cases (van den Boogaard et al. 2000). Two previous studies, reporting that 2% of cases of clefting had *MSX1* mutations, supported this hypothesis (Jezewski et al. 2003; Suzuki et al. 2004). Of the proposed pathogenic mutations, the P147Q variant was found in approximately 2% of Vietnamese (Suzuki et al. 2004) and 0.15% of Filipino cases (Vieira et al. 2005). In the current study, we used direct sequencing covering all the coding sequences of *MSX1* to determine whether *MSX1* mutations might be etiological in some cases of Thai patients with nonsyndromic CL/P.

Subjects and methods

The subjects of this study were 88 sporadic cases of nonsyndromic CL/P and 12 additional cases with a positive family history. Details of their characteristics and recruitment have been previously reported (Leoyklang et al. 2006). The study was approved by the institutional review board of the Faculty of Medicine of Chulalongkorn University, and written informed consent was obtained from each person included in the study. The control samples were Thai blood donors with no oral clefts who denied history of oral clefts in other family members.

Genomic DNA was isolated from peripheral blood, according to established protocols. Primers in noncoding regions were used to specifically amplify fragments encompassing coding regions in both exons (primers 1F and 1R for exon 1 and primers 2F and 2R for exon 2; Table 1) of the *MSX1* gene. Polymerase chain reactions (PCR) were carried out in a 20-μl volume containing 50 ng genomic DNA, 1× PCR buffer, 1.9–2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.5 U Taq polymerase, using the following parameters: 40 s at 94°C, 40 s at the annealing temperature (Table 1), and 40 s at 72°C for 35 cycles. PCR products were treated with ExoSAP-IT (USP, Cleveland, OH, USA) according to the manufacturer's recommendations, and sent for direct sequencing to Macrogen, Seoul, Korea. Primers used for sequencing were the same as those for PCR reactions, except the primer 1RS (Table 1), which was used for sequencing exon 1 in the 3′–5′ direction. Analyses were performed using Sequencher 4.2. When the results indicated a possible new variant, the sample was resequenced. The position of variants corresponds to the coding sequence for the nucleotide position within the Genbank entry AF426432.

The P147Q variant was verified in the patients by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using a mutagenesis primer P147Q-F and the primer 1RS (Table 1) and restriction endonuclease, *Dde*I. In addition, to determine whether the variant was associated with cleft lip in our Thai population, we performed an association study by genotyping 50 more patients with cleft lip with or without CL/P (bringing the total number of participants to 150) and 100 Thai controls.

Standard Chi-squared and p values were calculated by a program available at http://www.unc.edu/~preacher/chisq/chisq.htm. Odds ratio and 95% confidence intervals (95%CI) were calculated from the Epi Info 2000 program downloaded from http://www.cdc.gov/epiinfo/.

We determined whether the P147Q variant in the Thai population was on the same haplotype as the Vietnamese by typing three single nucleotide

Table 1 Oligonucleotides and polymerase chain reaction (*PCR*) conditions for *MSX1* mutation analysis. *SNP* single nucleotide polymorphism

Name	Primer sequences for PCR 5′–3′	Product size (bp)	Annealing temperature (°C)
1F	CCAGTGCTGCGGCAGAAGG	848	62
1R	ATTCATCCGCTGGGGTGAA		
2F	GGCTGATCATGCTCCAATGC	556	58
2R	CACCAGGGCTGGAGGAAT		
1RS	TGGAACCTTCTTCCTGGGTG	-	-
P147Q-F	CCGAGAGGACCCCGTGGATGCAGAGCCCCCGCTTCTCTC	(with primer 1RS) 231	58
G267C-R	CAGGAAACAGCTATGACCCTGGAAGGGGCCAGAGGCTC	(with primer 2F) 448	60
SNP1-F	TAGGGCTTCTCAGGGAATCA	230	55
SNP1-R	TTGCGTGGTTTCCCGTATAC		
SNP4/5-F	AAGTCCAAAGGATCGTTGTG	960	57
SNP4/5-R	GGGAAGATGTGAAATCACCT		

polymorphisms (SNPs), snp1, snp4, and snp5 (SNPs were designated in accordance with a previous study, Suzuki et al. 2004) using primers SNP1-F and SNP1-R (Table 1) and *BstB*I for snp1; primers SNP4/5-F and SNP4/5-R (Table 1) and *BseR*I for snp4; and primers SNP4/5-F and SNP4/5-R and *Mbo*II for snp5, in 50 Thai controls. Haplotype frequencies were estimated by the EH program, which tested and estimated linkage disequilibrium between different markers, downloaded from http://www.linkage.rockefeller.edu/ott/eh.htm.

Both of the novel nonsynonymous coding variants, 799G > T and 832C > T, were verified by PCR-RFLP, using the primer 2F and a mutagenesis primer G267C-R (Table 1) and *Dde*I for the 799G > T, and primers 2F and 2R and *Mwo*I for the 832C > T. One hundred and sixty-two Thai control individuals were also examined for the variants by restriction enzyme analysis.

For protein sequence comparisons, MSX1 orthologs were first identified through a BLAST search of the nonredundant database using Homo sapiens MSX1, accession NP_002439, as the reference sequence. All known and complete MSX1 sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by ClustalX 1.81 program. The human MSX1 was aligned with cow (accession NP_777223), Norway rat (accession NP_112321), house mouse (accession NP_034965), red jungle fowl (accession XP_444660), African clawed frog (accession AAH81101), and zebrafish (accession NP_571348). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

PolyPhen (http://www.bork.embl-heidelberg.de/PolyPhen/) was used to predict the effect of the nonsynonymous mutations. ESEfinder software (http://www.rulai.cshl.edu/tools/ESE/) was used to predict potential exonic splicing enhancers (ESEs; Shotelersuk et al. 2004).

Results

The sequencing effort concentrated on the coding regions of the *MSX1* gene. In 100 DNA samples from subjects with nonsyndromic CL/P, 8 variant sites were identified. Seven were single nucleotide changes, comprising 3 transitions (2 in coding regions) and 4 transversions (all 4 in a coding region). The other variant was a single nucleotide deletion in intron 1 (Table 2).

The coding regions of *MSX1* contained 6 different variants, 2 synonymous and 4 nonsynonymous. Two nonsynonymous variants, 101C > G (A34G) and 440C > A (P147Q), were in exon 1 and have been previously reported. The A34G variant, previously reported as a nonpathogenic polymorphism (Suzuki et al. 2004), was found in 8 of our patients, 4 were heterozygous and the other 4 were either homozygous or hemizygous. Individuals who were homozygous/hemizygous for A34G have never been reported previously. The P147Q variant, previously proposed to be a pathogenic missense mutation in Vietnamese and Filipino cases (Suzuki et al. 2004; Vieira et al. 2005), was found in 3 of our 100 patients, all in the heterozygous state. Because of its relatively high prevalence in our patient group, we determined its frequencies in 50 more patients with nonsyndromic CL/P and 100 normal Thai controls. The observed frequencies of the 440C and 440A alleles, and the CC and CA genotypes in affected subjects and controls are shown in Table 3. The observed distribution of genotypes among controls was compared with that expected according to the Hardy–Weinberg equilibrium: no difference was found ($\chi^2 = 0.007$, $df = 1$, $P = 0.93$). Genotype frequencies of the patients also followed the Hardy–Weinberg equilibrium ($\chi^2 = 0.002$, $df = 1$, $P = 0.96$). The distributions of alleles and genotypes among patients were compared with those among controls: no differences of either allelic ($P = 0.285$) or genotypic ($P = 0.277$) distributions between patients and controls were found.

Table 2 Variant sites of *MSX1* found in 100 Thai patients with nonsyndromic cleft palate (CL/P)

Nucleotide position	Exon/intron	Nucleotide change	Expected amino acid change	Frequencies of heterozygotes	Frequencies of homozygotes/hemizygotes
90	Exon 1	C > A	A30A	0	2
101	Exon 1	C > G	A34G	4	4
330	Exon 1	C > T	G110G	15	15
440	Exon 1	C > A	P147Q	3	0
452–14	Intron 1	del T	–	8	0
799	Exon 2	G > T	G267C	1	0
832	Exon 2	C > T	P278S	1	0
894 + 6	3′UTR	C > T	–	0	1

Table 3 Genotypic and allelic distributions and comparisons of the *MSX1* 440C > A in patients with CL/P and controls

	Patients (n = 150)	Controls (n = 100)	χ^2 (P value, df = 1)	Odds ratio (95% CI)
Alleles				
C	0.977	0.96		
A	0.023	0.04	1.145 (0.285)	
Genotypes				
CC	0.953 (143)	0.92 (92)		
CA	0.047 (7)	0.08 (8)	1.182 (0.277)	0.56 (0.18–1.78)

In addition, no association was found with the CA genotype compared with the CC (odds ratio 0.56, 95% CI 0.18–1.78; Table 3). In contrast to a previous study in a Vietnamese population (Suzuki et al. 2004), the P147Q variant was not associated with cleft lip in the Thai population. Next, haplotype analysis was performed in 50 unrelated control Thai. The results are shown in Table 4. Of the 8 440A alleles found in Thai controls, 7 were on the haplotype #5 (included in the haplotype #4 of a previous study, Suzuki et al. 2004), the same haplotype as in the Vietnamese. Due to the unavailability of samples from the relatives of our CL/P patients, we did not determine the haplotype of their P147Q variants.

The two nonsynonymous variants in exon 2, 799G > T (G267C) and 832C > T (P278S), have not been previously reported (Fig. 1). One variant was found in each of 2 patients; both were sporadic cases, with normal development, no anomalies besides the oral clefts, no consanguinity and no mutations in the coding region of *p63* (Leoyklang et al. 2006). Clinical and molecular features of these two patients with potential mutations are shown in Table 5.

Discussion

The sequencing analysis of the *MSX1* gene found four nonsynonymous variants. Two of them, the 799G > T (G267C) and 832C > T (P278S) variants, were predicted to be "probably damaging" by PolyPhen, changed (either created or eliminated) themselves as potential ESEs for serine-arginine (SR) proteins, and not present in 162 control individuals of Thai ethnic background. Both were found in exon 2 and have not been previously reported. Exon 2 of the *MSX1* was mostly conserved with significantly fewer sequence variations compared with exon 1 (Jezewski et al. 2003). These two potential mutations were found on the C-terminal side of the homeodomain protein while all of the previously reported potential missense mutations [E78V (Jezewski et al. 2003), G91D (Jezewski et al. 2003), G98E (Suzuki et al. 2004), V114G (Jezewski et al. 2003), G116E (Jezewski et al. 2003), and R151S (Jezewski et al. 2003)] were on the N-terminal side of the homeodomain protein. Moreover, for the P278S mutation, the proline 278 is conserved in MSX1 to the cow, rat, mouse, chicken, and frog protein sequence; and there is a substantial change in amino acid class, from nonpolar proline to polar serine.

Previous studies suggested that the P147Q variant is etiologically based on the strong conservation of the amino acid and the surrounding amino acids, the segregation analysis, and its absence in over 1,600 control individuals of various ethnic backgrounds (Suzuki et al. 2004; Vieira et al. 2005). Nevertheless, the fact that in some cleft families with the P147Q variant it was found in unaffected members while some affected did not carry it (Vieira et al. 2005) makes its role arguable. A previous observation suggested that it was a founder mutation in the Vietnamese population. In this study, the P147Q variant has been found in 8 out of 100 Thai controls, suggesting that it is not etiologic. In addition, an association between the variant and cleft lip in our population could not be detected (Table 3). Next, we determined whether the P147Q variant in our population was on the same haplotype as those in Vietnamese by genotyping three SNPs, which would be able to identify the four most common haplotypes in the Vietnamese (Suzuki et al. 2004). We did not genotype the snp2; therefore, haplotype #1 in

Table 4 Top 92% of haplotypes from 50 unrelated control Thai subjects

Haplotype # (designated in Suzuki et al. 2004)	Snp1[a] (−8796A > G)	P147Q (440C > A)	Snp4[a] (452–667T > G)	Snp5[a] (452–402G > T)	Frequency (#/total chromosomes)
1 (1 and 5)	A	C	T	T	27/100
2 (3)	G	C	T	T	27/100
3 (2)	A	C	G	G	22/100
4 (4)	A	C	T	G	9/100
5 (4)	A	A	T	G	7/100

[a]Numbers of SNPs are those used in Suzuki et al. (2004)

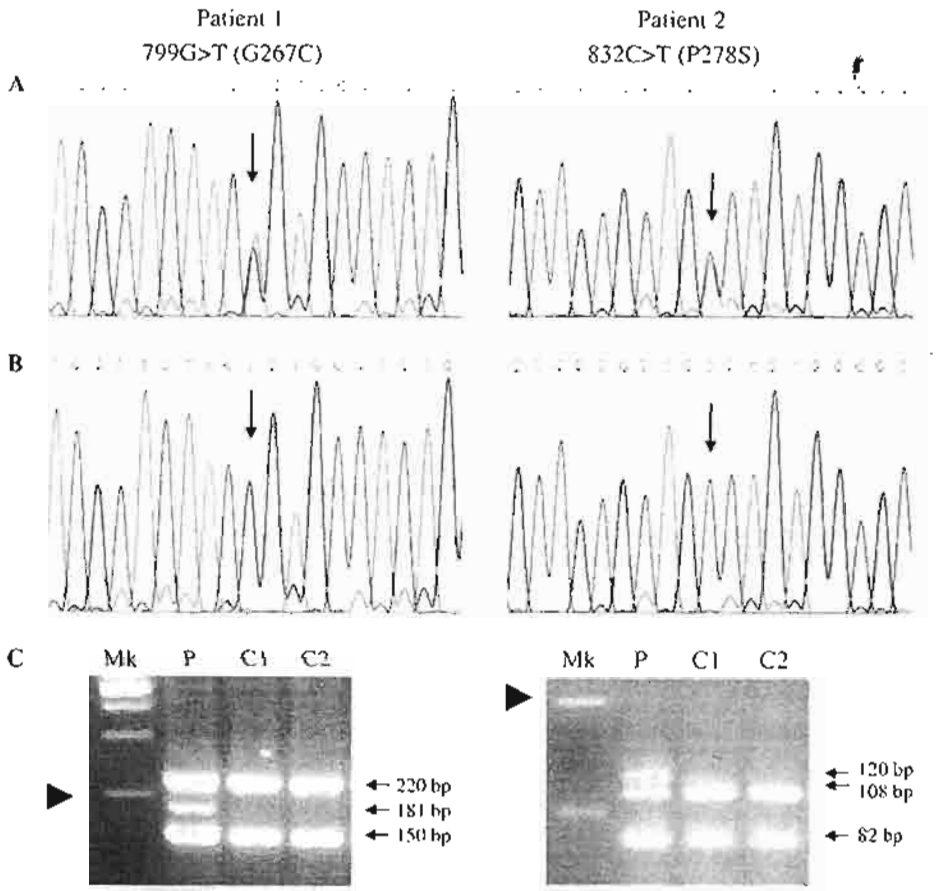

Fig. 1 Mutation analysis. The *left and right panels* relate to patients 1 and 2 respectively. **a** Electropherograms of patients, showing 799G > T and 832C > T (*arrows*) in patients 1 and 2 respectively. **b** Electropherograms of controls showing normal genotypes at nucleotide 799 as GG and 832 CC (*arrows*). **c** Restriction enzyme digestion analysis. C1 and C2 denote controls, Mk 100-bp marker, and *P* patient. The *arrowhead* indicates the 200-bp marker. In the *left panel*, *Dde*I digested the 448-bp product of controls into 220-, 150-bp, and other smaller products (not shown). The 799G > T mutation in patient 1 creates another *Dde*I restriction site. Therefore, the 220-bp PCR product of the mutant allele of the patient is further digested into 181- and 39-bp (not shown) products, present with the 220-bp product of the normal allele, indicating that patient 1 is heterozygous for the 799G > T mutation. In the *right panel*, *Mwo*I digested the 556-bp product of a control into 108-, 82-bp, and other smaller products (not shown). The 832C > T mutation in patient 2 eliminates a *Mwo*I site, leaving the 120-bp product, present with the digested 108- and 12-bp (not shown) of the normal alleles, indicating that patient 2 is heterozygous for the 832C > T mutation

Table 5 Clinical and molecular features of patients with potential mutations

	Patient 1	Patient 2
Age (years)	45	1.3
Sex	Female	Male
Province	Trang	Trang
Cleft type	Left complete cleft lip and palate	Left complete cleft lip
Nucleotide change (heterozygous)	799G > T	832C > T
Exon	2	2
Expected amino acid change	G267C	P278S
Animals with the same amino acid at the codon	Cow, chicken, frog[a]	Cow, rat , mouse, chicken, frog[a]
Position in MSX1	42 AA 3′ to homeobox	53 AA 3′ to homeobox
PolyPhen prediction	Probably damaging	Probably damaging
Frequency in 324 control chromosome	0	0

[a] Codon 267 of rat, mouse, and zebrafish is serine, which is a polar, uncharged amino acid; and codon 278 of zebrafish is asparagine, which is polar, uncharged

our study included both haplotypes #1 and #5 of the previous study (Suzuki et al. 2004). We showed that the P147Q variant in our control Thai subjects (Table 4) was on the same haplotype as those in Vietnamese (Suzuki et al. 2004). This observation strongly suggests that this P147Q variant in both the Thai and Vietnamese populations is inherited by descent from a founder genetic change. PolyPhen predicted the P147Q variant to be benign. This evidence suggests that the P147Q is neither pathogenic by itself, nor associated with cleft lip in the Thai population.

The A34G variant was a change within amino acid class, previously reported in cases and controls, and previously proposed to be benign (Jezewski et al. 2003; Suzuki et al. 2004). The roles of the two synonymous variants and the two variants in noncoding regions need further investigation.

Cleft palate is a multifactorial disorder caused by a combination of genes and environmental interactions. These factors may contribute differently to CL/P in different populations. In the Thai population, we have shown that there were associations between cleft lip and the maternal *MTHFR* 677CT/1298AC genotype, with an odds ratio of 4.43 and a 95%CI of 1.33–15.10 (Shotelersuk et al. 2003), and the *IRF6* 820G > A SNP of the proband, with an odds ratio of 1.67 and a 95% CI of 1.13–2.47 (Srichomthong et al. 2005). We also demonstrated that the *p63* mutation was responsible for approximately 1% of nonsyndromic cleft lips in the Thai population (Leoyklang et al. 2006). In this study, we have shown that *MSX1* mutations cause 2% of Thai cases of nonsyndromic cleft lip. Other populations may have different percentages of contributions from these genes.

This report demonstrates that the *MSX1* mutations appear to contribute about 2% of cases of nonsyndromic CL/P, consistent with two previous reports (Jezewski et al. 2003; Suzuki et al. 2004), but suggests that the P147Q variant is not pathogenic. Further studies of the full phenotypic spectrum and penetrance of *MSX1* mutations may improve genetic counseling in families with mutations.

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Research Letter

Tetralogy of Fallot With Absent Pulmonary Valve in a De Novo Derivative Chromosome 9 With Duplication of 9p13 → 9pter and Deletion of 9q34.3

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To the Editor:

The most common clinical manifestations of trisomy 9p syndrome include mental retardation, a wide fontanelle, microcephaly, downslanting and deep set eyes, prominent nasal root with a bulbous nasal tip, low-set abnormal ears, minor skeletal anomalies (hypoplastic phalanges, clinodactyly of the fifth finger and hypoplastic nails) and single palmar crease [Sutherland et al., 1976; Young et al., 1982; Haddad et al., 1996; Fujimoto et al., 1998; Sanlaville et al., 1999]. Intrauterine growth retardation, cleft lip/palate and congenital heart defect (CHD) are seen infrequently, unless the trisomic segments extend through 9q22–9q32 [Wilson et al., 1985].

In some cases of partial trisomy, analysis of the genotype–phenotype relationship is complicated by the presence of a complex chromosome rearrangement, which cannot be defined solely by conventional G-banding technique. Molecular cytogenetic methods, such as multicolor fluorescence in situ hybridization (mFISH) and multicolor banding (mBAND) analysis, may be required to define the interpretation [Chudoba et al., 2004]. Since small rearrangements involving chromosome end are not well represented in mFISH and mBAND, subtelomeric FISH probe sets can be applied to identify cryptic aberrations that might be the cause of dysmorphic features and idiopathic mental retardation in some patients [Knight et al., 2000].

We present a patient with clinical features resembling trisomy 9p in whom mFISH, mBAND and subtelomeric FISH demonstrated de novo trisomy 9p with an additional copy of 9p13 → 9pter attached to 9qter and a 9q34.3 subtelomeric deletion at the insertion breakpoint. While the 9p duplication explained several of the dysmorphic features, the 9q34.3 deletion might account for the conotruncal heart defects in this patient.

The proband was a female infant born at term, by vaginal delivery, to a 24-year-old, Gravida 0 Para 0 mother. Ultrasonography at 18 weeks gestational age showed polyhydramnios; and at 32 weeks gestational age a ventricular septal defect (VSD), pulmonary valve stenosis and tricuspid regurgitation were noted. Birth weight was 2,560 g (25th centile), length was 46 cm (25–50th centile), and head circumference was 30.5 cm (3rd centile).

Multiple dysmorphic features were noted at birth, including overlapping cranial sutures, bitemporal narrowing, sunken eyes with short and downslanting palpebral fissures, a bulbous nose, low-set ears, a right preauricular skin tag and over-folding of the

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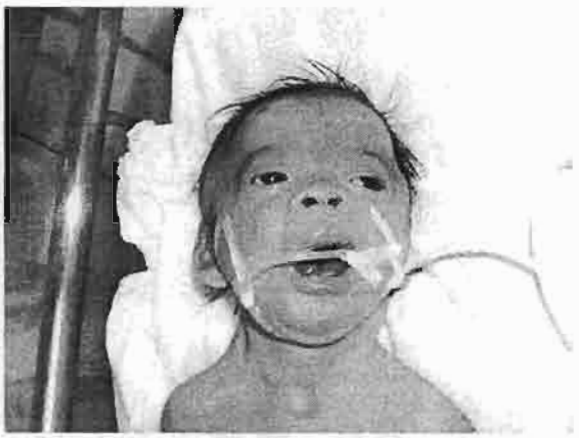

FIG. 1. Photograph of the patient at age 1 month. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

helices, redundant nuchal skin, webbed neck and a soft tissue mass at the midline of the anterior chest wall (Fig. 1). Also present were bilateral clinodactyly and hypoplasia of the middle phalanges of the fifth fingers and ulnar deviation of both thumbs. The toes were overlapping. The external genitalia appeared normal and there was a pilonidal sinus at the coccyx. Neurologically, the infant was hypoactive with hypotonia and normal neonatal reflexes.

Postnatal echocardiogram confirmed the prenatal findings of large VSD, atrial septal defect, patent ductus arteriosus and right ventricular hypertrophy consistent with Tetralogy of Fallot (TOF). Because the pulmonary valve leaflets were absent, annulus was hypoplastic and pulmonary trunk and proximal right and left pulmonary arteries were dilated, the heart was described as TOF with absent pulmonary valve syndrome. Family history was negative for CHDs and consanguinity. The patient died at age 4 months from cardio-respiratory failure related to the CHDs. Autopsy was declined.

Metaphase chromosomes were obtained from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes, and G-banding was performed using standard methods [Watt and Steven, 1986].

mFISH was performed on metaphase chromosomes using a 24XCyte probe kit (MetaSystems, Altlussheim, Germany) according to the manufacturer's instructions.

mBAND was performed on metaphase chromosomes using an XCyte9 chromosome 9-specific mBAND probe (MetaSystems). Both mFISH and mBAND images were captured and analyzed using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Jena, Germany) and the Isis software (MetaSystems).

The subtelomeric deletion of 9q was investigated by FISH using subtelomeric probes of 9p and 9q (BAC/PAC clones RP11-174M15 and RP11-885N19, respectively). Briefly, FISH was performed on metaphase chromosomes of the patient. Clones were directly labeled with SpectrumGreen or SpectrumRed (Vysis, Des Plaines, IL) by nick translation according to the manufacturer's specifications. Chromosomes were counterstained with DAPI.

TABLE I. Clones Used for 9qter Deletion and 9p13 Duplication Breakpoints Mapping

Clone 9q34.3	Location (Mb) from 9q telomere	Clone 9p21.1–13	Location (Mb) from 9p telomere
RP11–885N19	0.1	RP11–205M20	32.5
RP13–467E5	0.2	RP11–395N21	35.4
RP11–48C7	0.4	RP11–397D12	37.4
RP11–229P13	1.0		
RP11–413M3	1.4		
RP11–399H11	2.9		
RP11–374P20	4.1		

The locations of all clones correspond to the May 2004 draft sequence of the human genome on the UCSC Genome bioinformatics browser.

The 9q34.3 deletion and 9p duplication breakpoints were mapped by FISH in essentially the same manner using the clones listed in Table I.

Using the standard G-banding technique, the patient's karyotype was interpreted as 46,XX, der(9)t(9;?)(q34.3;?) at the 550-band level of resolution (Fig. 2). The derivative chromosome 9 had extra material attached to the 9qter. Both the paternal and maternal karyotypes were normal, suggesting a de novo event.

mFISH confirmed that the der (9) extra material was of chromosome 9 origin (Fig. 3A–C). mBAND analysis revealed an additional copy of 9p13 → 9pter attached to the 9qter in a direct fashion, resulting in partial trisomy of 9p (Fig. 3D and 4). To determine the presence of 9q subtelomeric region of the derivative chromosome, FISH using 9p and 9q subtelomeric probes was carried out. The results demonstrated a subtelomeric deletion at the insertion breakpoint on the 9qter (Fig. 5A). Mapping of the 9q34.3 deletion breakpoint was accomplished by systematically narrowing by FISH with genomic clones located within the most distal 4 Mb of 9qter. The 6 BAC clones used in mapping the deletion breakpoint and their locations from 9q telomere are listed in Table I. FISH demonstrated the deletion breakpoint lies between clone RP11–413M3 and RP11–229P13 (Fig. 5B,C). The deletion size was approximately 1.4 Mb. The 9p duplication breakpoint was mapped by FISH using clones spanning region 9p21.1–9p13 listed in Table I. The result indicated that the duplication size was about 35.4 Mb (Fig. 5D).

This patient with multiple dysmorphic features, TOF with absent pulmonary valve, and an abnormal karyotype showing a derivative chromosome 9 with an abnormal long arm was more precisely defined using mFISH and mBAND. The results revealed an unusual case of de novo trisomy 9p in which an additional copy of the 9p13 → 9pter was inserted at

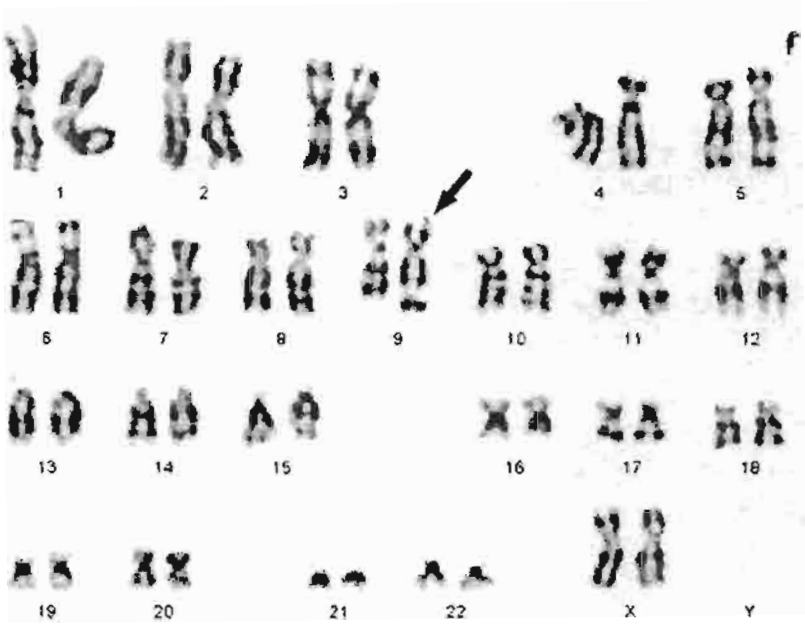

FIG. 2. Karyotype of G-banded chromosomes, showing the normal chromosome 9 and the derivative chromosome 9 (arrow).

9qter. Although the distal half of the short arm of chromosome 9 (9p13 → 9pter) is responsible for the major clinical features of trisomy 9p [Fryns et al., 1979; de Pater et al., 2002], CHDs, including conotruncal defects, are uncommon [Tennstedt et al., 1999; Morrissette et al., 2003]. Subtelomeric FISH demonstrated that there was a cryptic subtelomeric deletion at the insertion breakpoint

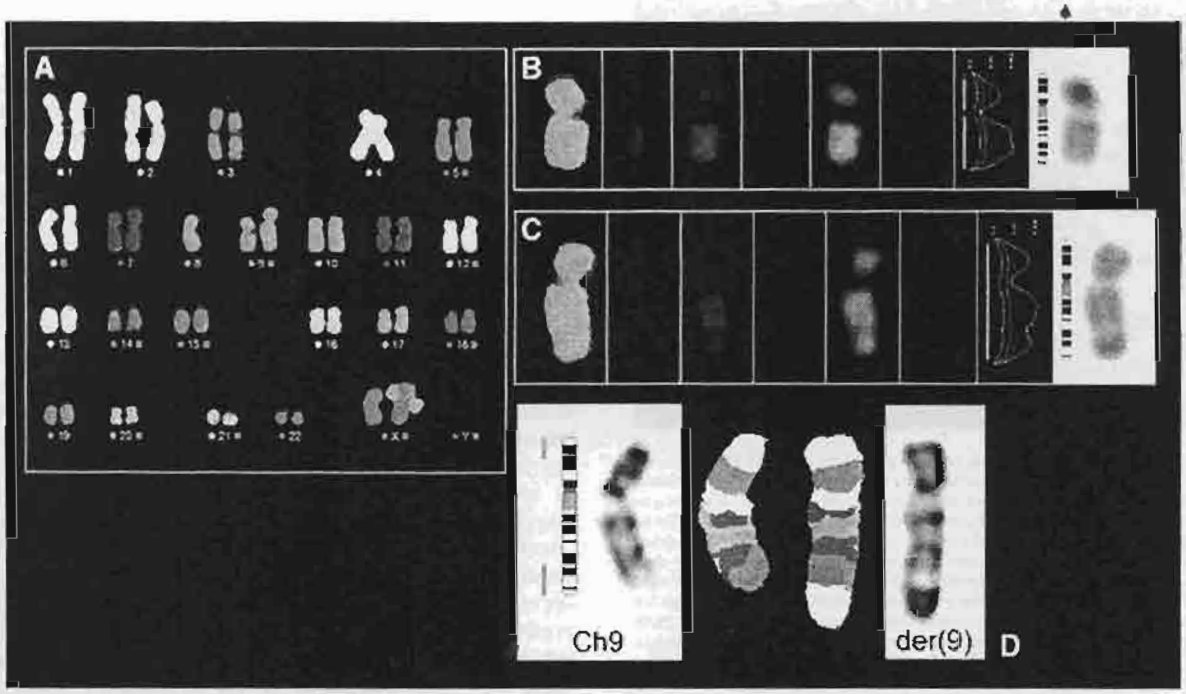

FIG. 3. A: Karyotype of mFISH confirmed that the extra material of the derivative chromosome 9 was all of chromosome 9 origin (B and C). D: mBAND showed the duplicated 9p13 → pter inserted to the 9qter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

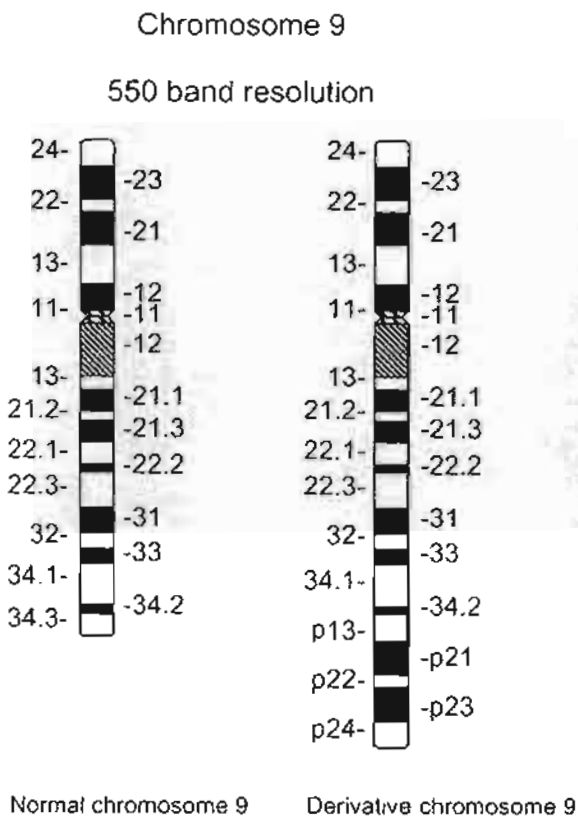

FIG. 4. Ideogram illustrates the duplicated segment of the short arm and the insertion breakpoint at the terminal end of the long arm of the derivative chromosome 9.

(9q34.3) on the 9qter, which might explain the CHDs in this patient.

Fine mapping of the 9q34.3 deletion breakpoint indicated that the deletion size was approximately 1.4 Mb, corresponding to the critical chromosomal region responsible for subtelomeric 9q34.3 deletion phenotypes [Stewart et al., 2004]. This minimum critical region is an approximately 1.2 Mb interval from the 9q telomere, encompassing at least 14 genes or transcripts, and it is suggested that haploinsufficiency of one or more genes within this region most probably accounts for the 9q- syndrome. Recent study suggested that *EHMT1* gene, which locates in this commonly deleted region, was most likely responsible for the larger part of 9q- phenotype [Kleefstra et al., 2005; Kleefstra et al., 2006]. A review of the literature showed that the phenotypic findings of the subtelomeric deletions of chromosome 9q34.3 generally consist of mental retardation, distinct facial features and CHDs particularly conotruncal heart defects [Iwakoshi et al., 2004; Stewart et al., 2004; Neas et al., 2005]. Table II summarizes the clinical features in 25 reported patients with subtelomeric 9q34.3 deletions compared to the clinical features of partial trisomy 9p patients. Our patient showed the characteristic face and hands of the trisomy 9p syndrome while the features commonly seen in 9q34.3 deletion patients such as mid-facial hypoplasia with synophrys and/or arched eyebrows.

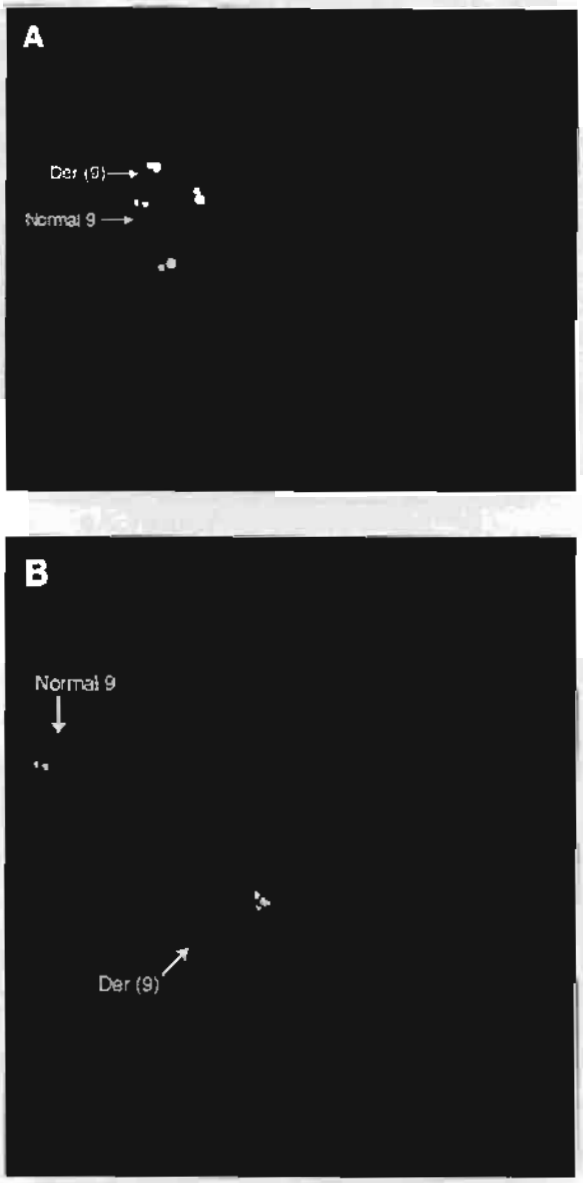

FIG. 5. FISH mapping of the 9q deletion and 9p duplication breakpoints. A: Metaphase spreads were analyzed for the presence of subtelomeric region of the long arm of the derivative chromosome 9 at the insertion breakpoint by FISH using subtelomeric probes. The green signals indicated the subtelomeric region of the 9p and the red signal was the subtelomeric region of the 9q. The 9q subtelomeric region of the der(9) was found to be deleted at the insertion breakpoint. B: Clone RP11–413b13 was labeled as green and RP11–374P20 was labeled in red. Both green and red signals could be detected on both chromosome 9 and der(9). C: Clone RP11–229P13 and RP11–393H3 were labeled in green and red, respectively. Red signals could be detected on both chromosome 9 and der(9), whereas green signal could not be detected on der(9). The results indicated that the deletion breakpoint lies between BAC clone RP11–229P13 and RP11–413b13. D: Clone RP 11–205M20 and RP11–395N21 were labeled in red and green, respectively. The result showed an additional red signal indicative of duplication on der(9) (arrow). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

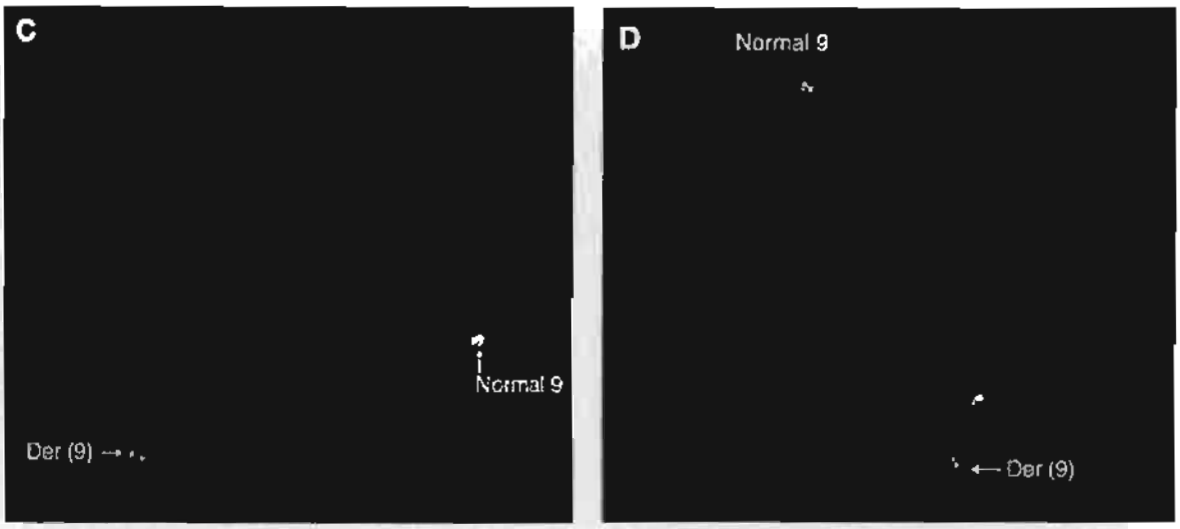

FIG. 5. (*Continued*)

TABLE II. Clinical Manifestations of Our Patient Compared With Partial Trisomy 9p and 9q34.3 Deletion

Clinical manifestation	Partial trisomy 9p[a] p13 → pter (%)	9q34.3 deletion[b] (%)	Our case
Craniofacial			
Microcephaly	8/24 (33)	23/25 (92)	+
Brachycephaly	7/24 (29)	12/25 (48)	+
Flat face		5/25 (20)	−
Hypoplastic midface		13/25 (52)	−
Arched eyebrows		8/25 (32)	−
Deep-set eyes	7/24 (29)		+
Hypertelorism	10/24 (42)	12/25 (48)	+
Down-slanting palpebral fissures	9/24 (37.5)	6/25 (24)	+
Bulbous nose	14/24 (58)		+
Short nose		14/25 (56)	−
Anteverted nostrils		9/25 (36)	−
Carp mouth		20/25 (80)	−
Thin upper lip		6/7 (86)	−
Thick lower lip		5/25 (20)	−
Down-turned corners of the mouth	13/24 (54)		+
Protruding tongue		12/25 (48)	−
Low-set ears	10/21 (48)	3/5 (60)	+
Malformed ears	19/24 (79)	12/25 (48)	+
Musculoskeletal			
Short or webbed neck	7/24 (29)		+
Clinodactyly	19/24 (79)		+
Single palmar transverse crease	16/24 (67)	5/6 (83)	+
Syndactyly	1/24 (4)	2/6 (33)	−
Hypoplasia of phalanges	20/24 (83)	4/22 (18)	+
Dysplasia or hypoplasia of nails	11/24 (46)		+
Congenital heart defects[c]			
Total	1/24 (4.2)	12/25 (48)	
Conotruncal defects		3/12 (25)	TOF with absent pulmonary valve
Left-sided obstruction		1/12 (8.3)	
Atrial and ventricular septal defects		8/12 (66.7)	
Valvular defects	1/1 (100)		

[a]Number positive/number informative. Frequencies are derived from cases in Lewandowski et al. [1976], Fryns et al. [1979], Bussani Mastellone et al. [1991], Phelan et al. [1993], Haddad et al. [1996], Fujimoto et al. [1998], Tsezou et al. [2000], and de Pater et al. [2002].

[b]Number positive/number informative. Frequencies are derived from cases in Iwakoshi et al. [2004], Stewart et al. [2004], Neas et al. [2005], and Kleefstra et al. [2006].

[c]Additional CHD types were not observed.

anteverted nares, carp shape mouth, thick lower lip, and tongue protrusion, were not present (Table II). TOF with absent pulmonary valve in our patient was more compatible with the conotruncal CHDs associated with 9q34.3 deletion [Stewart et al., 2004]. It is possible that the subtelomeric region of 9q might be critical for heart development, especially for conotruncal development.

In most reported cases, the partial trisomy 9p was the result of a parental reciprocal translocation between chromosome 9 and another chromosome; in a small number, it was due to the tandem duplications [Cuoco et al., 1982; Haddad et al., 1996; Fujimoto et al., 1998; Tsezou et al., 2000; Christina et al., 2003]. In this new patient, the duplicated 9p segment (9p13 → 9pter) was inserted at the terminal end of the long arm, and the subtelomeric region of 9q34.3 was found to be deleted. One possible mechanism to explain how this unusual cytogenetic finding occurred is the telomere capture, through which a terminally deleted chromosome is stabilized by acquiring a new telomeric sequence from another chromosomal location, which results in a derivative chromosome [Ballif et al., 2004]. However, such a chromosome abnormality was not typical of cases of de novo trisomy 9p and could not be confirmed by the G-banding technique alone.

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Cancer Genetics Report

PTEN c.511C>T Nonsense Mutation in a BRRS Family Disrupts a Potential Exonic Splicing Enhancer and Causes Exon Skipping

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Bannayan–Riley–Ruvalcaba syndrome (BRRS) is an autosomal dominant disorder characterized by macrocephaly, intestinal hamartomatous polyps, lipomas and pigmented macules of the glans penis. We identified a Thai family affected with BRRS. In addition to typical manifestations of BRRS, the proband has a large hepatic AVM which is rarely found in BRRS. The molecular analysis revealed affected members were heterozygous for an exon skipping-associated nonsense mutation c.511C>T in the *PTEN* gene. The mutation was previously assumed to be deleterious by causing a change to a termination codon, Q171X. We, herein, found that another pathogenic effect was splicing related by disrupting a potential exonic splicing enhancer (ESE) and causing an entire exon 6 skipping. The results prompted us to investigate other reported missense/nonsense mutations in the *PTEN* gene. We found that they do not colocalize with ESE sites, suggesting that most of their pathogenic effects are not through ESE disruption.

Key words: Bannayan–Riley–Ruvalcaba syndrome – Cowden syndrome – nonsense mutations – exonic splicing enhancer

INTRODUCTION

Germline mutations in the tumor suppressor gene *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) have been implicated in PTEN hamartoma tumour syndromes (PHTS) including Cowden syndrome (CS; MIM 158350), Bannayan–Riley–Ruvalcaba syndrome (BRRS; MIM 153480), Proteus syndrome and Proteus-like syndrome (1–4). Although the clinical manifestations of these four disorders differ significantly, all have increased likelihood of benign tumor growth. Of the four entities, CS and BRRS display the highest degree of clinical overlap. CS is an autosomal dominant disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and an increased risk of breast, thyroid, and endometrial and other cancers (5–8). BRRS, a phenotypic variant of CS, is characterized by the cardinal features of macrocephaly, intestinal hamartomatous polyps, lipomas and pigmented macules of the glans penis. Other features in BRRS include Hashimoto's thyroiditis, vascular malformations, hemangiomas and mental retardation (9–11).

Approximately 60–65% of individuals with a clinical diagnosis of BRRS have a detectable *PTEN* gene mutation (1,12–14). The *PTEN* gene maps to 10q23.3 and encodes a dual-specificity phosphatase which antagonizes the phosphoinositol-3-kinase (PI3K)/Akt pathway leading to G1-cell-cycle arrest or apoptosis as well as inhibits cell spreading via the focal adhesion kinase pathway (15–19). Genotype–phenotype analyses revealed an association between the presence of *PTEN* mutation in BRRS and the development of lipomas and tumors of the breast (1). Individuals with BRRS and *PTEN* mutations are currently thought to have the same cancer risks as individuals with CS. Therefore, it has been suggested that individuals with BRRS and *PTEN* mutation should receive equal attention with respect to cancer surveillance (1).

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At least 173 different disease-causing mutations have been characterized in the *PTEN* gene (http://www.hgmd.cf.ac.uk). Of these, 75 are missense/nonsense mutations. Identical mutations have been identified in some families with Cowden syndrome and in others with BRRS. In addition, families whose members have overlapping features of both conditions have been identified (1,14,20). There is still no clear explanation for this phenomenon.

We present a Thai BRRS family with a germline mutation, c.511C>T (p.Q171X), in the *PTEN* gene. The mutation has never been reported in BRRS. In addition, our patient has a large AVM in the liver which is rarely found in BRRS. We also demonstrated that this nonsense mutation actually disturbed splicing presumably from disrupting a potential exonic splicing enhancer (ESE) causing skipping of the whole exon 6. In addition, we have found that other reported missense/nonsense mutations in the *PTEN* gene do not colocalize with ESE sites, suggesting that most of their pathogenic effects are not through ESE disruption.

PATIENTS AND METHODS

Clinical Report

A 48-year-old Thai male patient presented with a 5-day history of painless hematochezia. He denied any fever, nausea, vomiting, abdominal pain and weight loss. He rarely drinks or smokes. The gastroscopy revealed three medium size esophageal varices at distal esophagus, multiple polyps starting from hypopharynx to lower esophagus, in gastric remnant, duodenum and jejunum. Colonoscopy showed multiple sessile polyps in his entire colon and rectum (more than 100 polyps). Their sizes varied from 0.5–1.5 cm. The biopsy showed hamartomatous polyps.

His past medical history was notable for multiple episodes of gastrointestinal bleeding. When he was 15 years old, he was hospitalized for upper GI bleed. He underwent gastroscopy which revealed multiple gastric polyps. He also developed several subcutaneous nodules of various sizes on his trunk and extremities since age 10. He had undergone several surgical resections of subcutaneous lipomas.

His birth history was unavailable. The family history was significant for multiple subcutaneous masses in his mother and his second daughter. Even though his mother was noted to have multiple subcutaneous masses and now is 80 years old, she has never suffered from major illnesses. She was unavailable for evaluation. His 21-year-old elder daughter has not had any clinical features of BRRS. His second daughter is 17 years old. According to her mother, she was born at term after an uncomplicated pregnancy with birth weight 4000 g (>97th centile). Other birth parameters were unavailable. Her development was appropriate for age. She was diagnosed with hypothyroidism at age 9. She also developed multiple subcutaneous masses of various sizes on all four extremities and trunk since age 9. The proband also has 10 other siblings. They were noted to have no clinical features of BRRS and unavailable for evaluation. The pedigree of this family is shown in Fig. 1A.

At the first genetic evaluation of the proband, physical examination revealed a height of 1.75 m (25th centile), weight of 60 kg (15th centile) and head circumference of 61 cm (>97th centile). A small purplish nodule was noted at his right buccal mucosa. The thyroid gland was not enlarged on palpation. Abdominal examination revealed an old surgical scar with centrifugal superficial vein dilatation. His abdomen was moderately distended with positive shifting dullness. His liver and spleen were not enlarged. Multiple subcutaneous nodules of various sizes were seen on his trunk and all four extremities. Examination of the genital region showed pigmented macules of the glans penis (Fig. 1B and C).

Abdominal CT scan showed hypodensity lesion at left lobe of the liver, splenomegaly and ascites (Fig. 2A). Angiogram was performed and showed a large arteriovenous malformation at left hepatic lobe with enlarged left hepatic artery (Fig. 2B and C). A rapid draining into dilated portal vein was noted. Embolization of the feeding branches was performed three times with less than 20% residual shunt remained.

Physical examination of the proband's second daughter showed a height of 1.71 m (95th centile), weight of 63 kg (50th centile). There were no dysmorphic features.

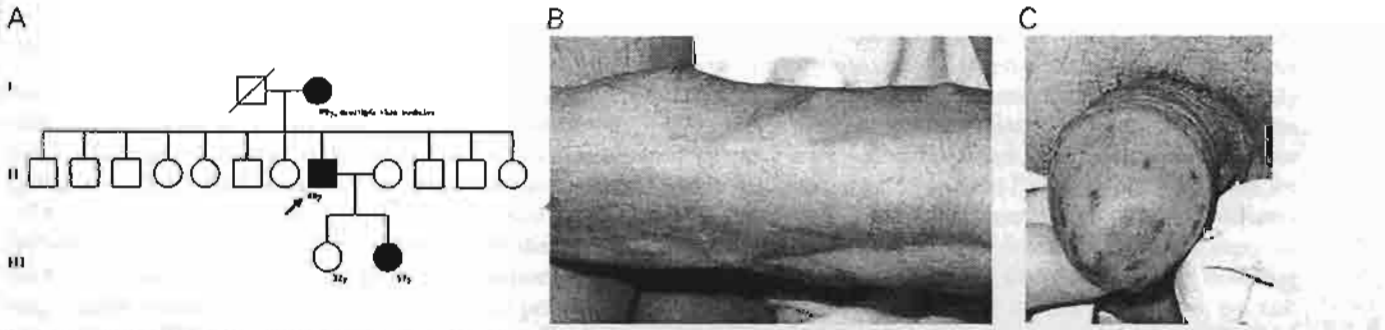

Figure 1. Pedigree of a BRRS family (A) and photograph of the proband showing multiple subcutaneous lipomas over the upper extremity (B) and pigmented macules of the glans penis (C). (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

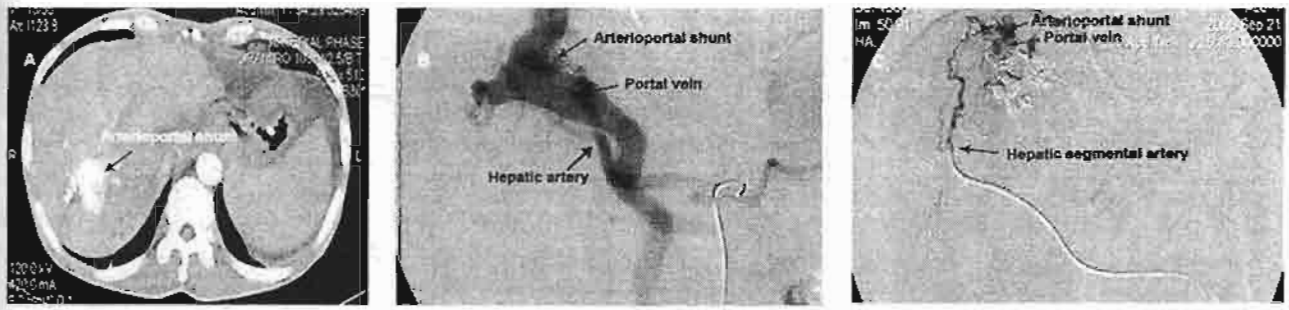

Figure 2. Imaging of the hepatic arteriovenous malformation in the proband. (A) Computed tomographic scan of the upper abdomen showing hepatic arterioportal shunt. (B, C) Angiogram of the hepatic arteriovenous malformation showing a large high flow arterioportal shunt from hepatic arteries to portal vein with marked enlargement of portal vein. (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

Table 1. Summary of genetic features of the proband

Characteristics	Variable
Ethnicity	Thai
Gene	*PTEN*
GenBank accession No.	U93051, U96180, U92436
Chromosome assignment	10q23
Type of DNA variant	Germline nonsense mutation
Mutation	A C → T substitution in exon 6 at nucleotide 511 resulting in a change from glutamine to a stop codon (Q171X) as well as a partial exon 6 skipping causing a frameshift with a stop codon 9 amino acids downstream
Allelic frequency	–
Method of mutation detection	Direct sequencing

The thyroid gland was not enlarged. Multiple subcutaneous nodules of various sizes were seen on her trunk and bilateral forearms and legs. Endoscopy and colonoscopy were performed and revealed multiple small sessile polyps (0.3–0.5 cm in size) in esophagus, duodenum, terminal ileum, sigmoid colon and rectum. Genetic features of BRRS and the proband are summarized in Table 1.

PTEN Gene Analysis

After informed consent was received, 3 ml of peripheral blood from the patient and his two daughters were obtained. Total RNA was isolated from white blood cells using QIAamp® RNA blood mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using ImProm-II™ reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. PCR amplification of the *PTEN* cDNA exons 1–9 was performed using primers PTENF1 and PTENR1 as shown in Table 1. We used 2 μl of first-strand cDNA, 1 × PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 0.1 U Taq DNA polymerase (Promega) in a total volume of 20 μl. PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH, USA), according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. Genomic DNA of the proband and his daughters was extracted from peripheral blood lymphocytes using standard techniques. The 3′ end of exon 5, the whole exon 6, and the whole exon 7, including flanking intronic sequences of the *PTEN* gene were amplified using primers PTENEx5-F, PTENEx5-R, PTENEx6-F, PTENEx6-R, and PTENEx7-F, PTENEx7-R respectively as shown in Table 2. PCR was performed in 20 μl, containing 2 μl of genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer and 0.1 U Taq DNA polymerase. The products were directly sequenced as above. RTPCR was performed using primers PTENF2, PTENR2 to amplify the *PTEN* cDNA exons 5–8. All the PCR conditions were shown in Table 2.

Study of Association of Pathogenic Missense/Nonsense Mutations and ESE Sites in the *PTEN* Gene

Published data on pathogenic missense/nonsense mutations of the *PTEN* gene were used. We searched the coding regions of the *PTEN* gene for the presence of ESE motifs with ESEfinder software (http://rulai.cshl.edu/tools/ESE/) (21). To lower the number of false-positive results, we used a more stringent than recommended threshold value of 3.0 for all four types of ESE motifs as recommended in a previous study (22). ESEs in exon/exon boundaries were excluded as these sequences were separated by an intron. For an association study of nonsense mutations and ESE sites, we calculated the percentages of sequences that were potential ESEs, which provided us with the proportion of nonsense mutations expected to be in ESE motifs. We, then, classified reported nonsense mutations into those in ESE motifs and those do not. Comparison of the observed and expected frequencies of the mutations in ESE sites was performed using standard χ^2 and

Table 2. Primers and PCR conditions

Primers	Sequences	Annealing temperatures (°C)
PTENF1	5′-AAGTCCAGAGCCATTTCCAT-3′	61
PTENR1	5′-GACACAATGTCCTATTGCCA-3′	
PTENF2	5′- TGAAGACCAT AACCCACCAC-3′	55
PTENR2	5′-CCTTGTCATTATCTGCACGC-3′	
PTENEx5-F	5′-GATCTTGACCAATGGCTAAG-3′	53
PTENEx5-R	5′-CACAATGTATATACACATACATC-3′	
PTENEx6-F	5′-ATGTTCTTAAATGGCTACGAC-3′	55
PTENEx6-R	5′-AACCCATTGCTTTTGGCTTC-3′	
PTENEx7-F	5′-GATTGCAGATACAGAATCCAT-3′	55
PTENEx7-R	5′-TAATGTCTCACCAATGCCAG-3′	

P-value by a program available at http://www.unc.edu/~preacher/. For the association study of missense mutations and ESE sites, we performed similar analysis.

RESULTS

RNA was prepared from leukocytes of the proband and reverse transcribed. The cDNAs were then sequenced. The chromatogram showed two transcripts with different height. The proband was heterozygous for a mutation in the *PTEN* gene causing a skipping of the whole 142-bp exon 6 (Fig. 3B). This skipping is predicted to result in alterations starting from codon 165 and leading to a frameshift with a stop codon at position 174. The premature termination codon (PTC) usually triggers nonsense-mediated mRNA decay. This mechanism is likely to result in a reduction of PTC-harboring mRNA in our patient as shown in Fig. 3A and B. The genomic DNA of the relevant regions of the proband and his affected daughter was amplified by PCR and directly sequenced. The sequences revealed that they were heterozygous for c.511C>T presumably producing a stop codon (p.Q171X) (Fig. 3D). We further went through all the rest of the sequenced cDNA. The corresponding mutation found in the genomic DNA was also identified as a small peak in the chromatogram with the other two transcripts, the wild type transcript and the degraded frameshift cDNA resulting from an exon 6 skipping (Fig. 3E). No other mutations were identified in *PTEN* cDNA and genomic DNA of the proband. RT-PCR to amplify exon 5 to exon 8 revealed two bands of different sizes and amount in the proband (Fig. 3A). A reduced amount of exon-skipped transcript harboring PTC is most likely due to an incomplete penetrance of the exon 6 skipping as well as nonsense-mediated mRNA decay. RNA and genomic DNA of the unaffected elder daughter were also included in the study (Fig. 3C and F).

Based on the assumption that mutation-associated exon skipping has been mostly associated with ESE disruption, we investigated whether the c.511C>T mutation lies in and abrogates a high score ESE motif in the region encompassing the mutated residue. We analyzed the wild-type and mutant *PTEN* exon 6 sequences with two available ESE-prediction softwares, ESEfinder (21) and RESCUE-ESE (23). The results revealed that, although slightly reducing the score of an SRp40 motif from 4.6 to 4.3 without falling below the threshold value, the c.511C>T mutation eliminates a potential ESE of an SF2/ASF motif from 3.53 to 0.582 (Fig. 4).

Potential ESE motifs found in the *PTEN* gene are listed in Table 3. After excluding ESEs in exon/exon boundaries, we found potential ESE motifs encompassing 390 bp out of the 1212 bp (32.2%) of *PTEN* coding region. Of the 28 reported nonsense mutations, nine are in ESE (32.1%). Using the χ^2 test, we have found that nonsense mutations do not colocalize with ESEs ($\chi^2 = 0$, df = 1, $P = 0.99$). For analysis of missense mutations, of the 47 reported pathogenic missense mutations, 15 are in ESE (31.9%). Using the χ^2 test, we have also found that missense mutations do not colocalize with ESEs ($\chi^2 = 0.001$, df = 1, $P = 0.97$).

DISCUSSION

Our patient has clinical features including vascular malformation suggestive of BRRS. Hemangiomas and AVMs are recognized features of BRRS. There are occasional reports of large visceral AVMs. However, there has been no prior report of BRRS with AVMs in the liver. The pathogenesis of the AVM in this syndrome is still under investigation. There is growing evidence that PTEN is involved in modulating angiogenesis. PTEN is expressed in vascular smooth muscle cells and has an antiangiogenic effect (24,25). It has been shown that PTEN down-regulates new vessel formation through suppression of vascular endothelial growth factor (VEGF) expression (26). These findings suggest that loss of PTEN function leads to dysregulation of

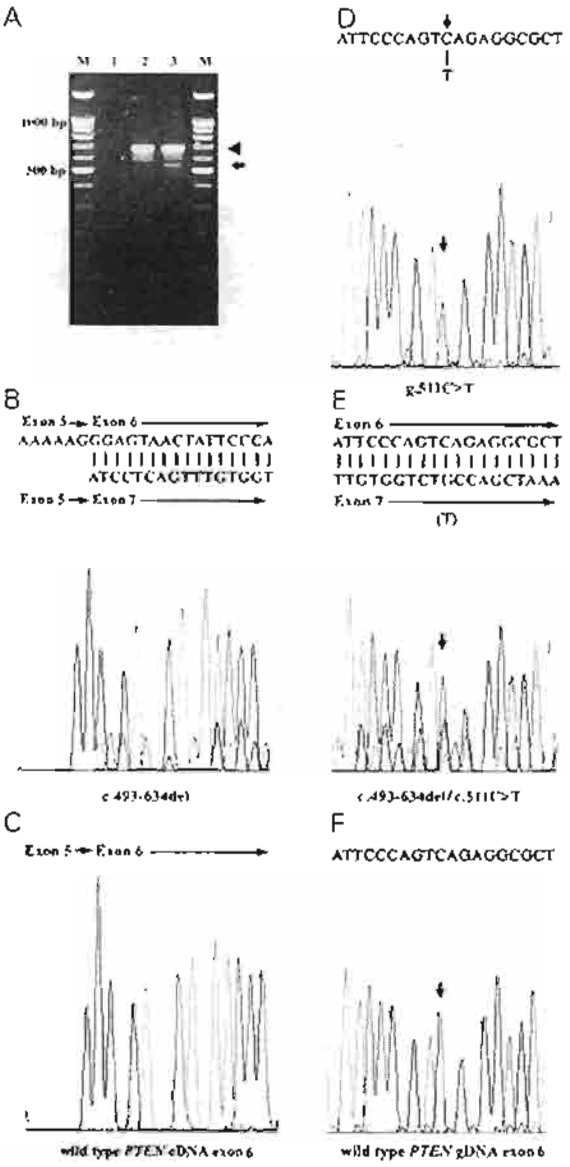

Figure 3. The *PTEN* gene analysis. (A) RT-PCR using primers PTENF2 and PTENR2 to amplify exons 5–8 of the *PTEN* cDNA. M: 100-bp marker. The 500 and the 1000-bp bands were indicated. 1, no cDNA; 2, unaffected control; 3, proband. There was an expected 671-bp band (arrowhead) from both control and the proband. A 529-bp product with less intensity was also found in the proband (arrow). The mutation found in the proband is likely to cause skipping of the whole 142-bp exon 6. (B) Partial sequences of the cDNA of the proband showing two transcripts with different height. The higher representing the wild type transcript whereas the lower representing the transcript with skipping of the entire exon 6. (C) Partial sequences of the cDNA of the unaffected elder daughter. (D) gDNA of the proband showing a C → T substitution in exon 6 at nucleotide 511 (arrows). (E) The further sequences of the cDNA of the proband showing the corresponding nucleotide substitution found in the gDNA as indicated by the arrow in addition to the wild type exon 6 transcript and the degraded exon-skipped transcript. (F) gDNA of the unaffected elder daughter showing only C in exon 6 at nucleotide 511 (arrow). All the shown chromatograms were in the 5′ to 3′ direction. (Please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org)

angiogenesis, a possible mechanism underlying AVM in this syndrome.

His cDNA was sequenced and revealed that he was heterozygous for a mutation in the *PTEN* gene causing a skipping of the whole 142-bp exon 6 (c.493-634del). In order to identify the basis of exon skipping, we amplified and sequenced the gDNA of the proband and his affected daughter from the 3′ end of exon 5 to the 3′ end of exon 7. To our surprise, we observed no mutation in the studied *cis*-acting consensus elements including flanking intronic sequences as well as a possible branch site in intron 5 (TTTCAAT) known to be involved in RNA splicing. However, a C → T substitution was found in exon 6 at nucleotide 511, which is expected to change a glutamine to a stop codon (Q171X) leading to the truncation of 233 amino acids from the PTEN protein. This corresponding nonsense mutation with very low magnitude was identified in the chromatogram of the sequenced cDNA suggesting a nonsense-mediated mRNA decay. This nucleotide substitution also caused a partial exon 6 skipping from a mutation causing ESE disruption. The fact that we found the transcript with exon 6 skipping resulting in frameshift leading to truncated protein product in this patient suggested another pathogenic effect of g.511C>T (Q171X). A reduced amount of the exon-skipped transcript harboring PTC is likely due to nonsense-mediated mRNA decay. Here we showed that ESE disruption causing an exon 6 skipping might be one of the pathogenic effects of c.511C>T mutation in the BRRS family. Further studies to show the c.511C>T mutation actually disrupts an SF2/ASF motif by using a mini gene construct and RNA-protein binding experiments will provide a strong evidence supporting this finding.

ESEs are discrete, degenerative motifs of 6–8 nucleotides located inside exons. The study of normal splicing suggests that most exons contain at least one functional ESE site. ESEs are required for definition and/or efficient splicing of the exons in which they reside. ESEs are target sequences for the family of conserved essential splicing factors—the serine/arginine-rich (SR) proteins (27). Nucleotide substitution in ESEs can result in decreased binding of SR proteins or other splicing factors to the ESE, leading to a failure to recognize the sequence as exon by the spliceosome and to exon skipping. Splicing signals are a frequent target of mutations in genetic diseases and cancer. It has been estimated that at least 15% of point mutations that result in a human genetic disease cause RNA splicing defects (28). We analyzed the wild type and mutant *PTEN* exon 6 sequences with ESEfinder software and found that the c.511C>T mutation eliminated the potential ESE of SF2/ASF motif. This result suggested that the c.511C>T mutation disrupted an ESE motif and caused an entire exon 6 skipping. This skipping is predicted to result in alterations starting from codon 165 and leading to a frameshift, causing a degradation of PTC-harboring mRNA.

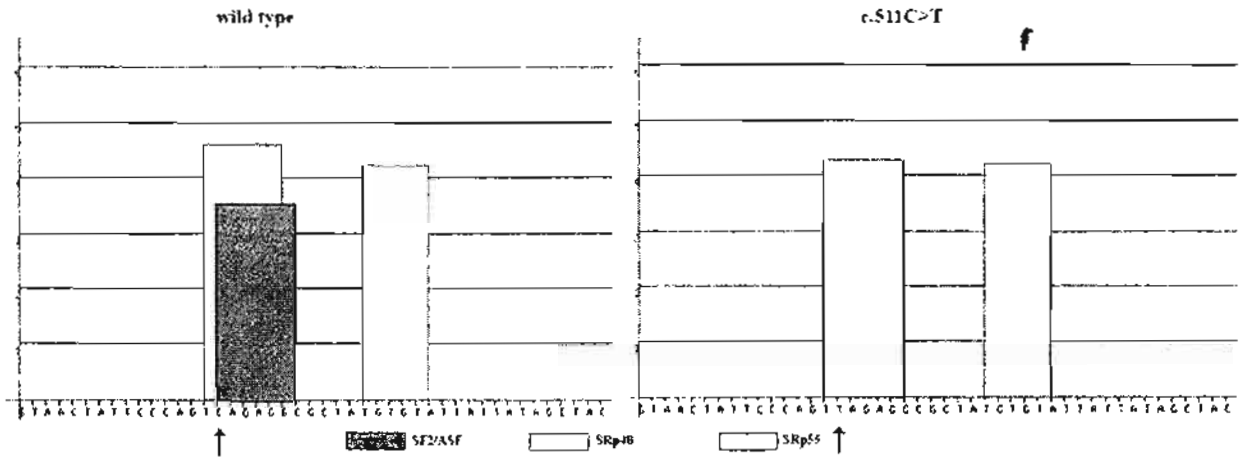

Figure 4. Effect of the c.511C>T mutation on SR proteins matrix score identified by ESEfinder software. The c.511C>T mutation eliminates the potential ESE of SF2/ASF motif from 3.53 to 0.582 and slightly reduces the score of an SRp40 motif from 4.6 to 4.3 without falling below the threshold value. The SC35 and SRp55 motifs are unchanged. The arrows indicate C in the wild type and T in the mutant.

At least 173 different mutations have been reported in the *PTEN* gene. Of these, 28 are nonsense mutations. Most mutation-screening studies are only conducted on genomic DNA. Q171X has already been reported one time in Cowden disease on genomic DNA (http://www.hgmd.cf.ac.uk), so the skipping of exon 6, which is the main pathogenic effect of this mutation, was not investigated. To our knowledge, the c.511C>T mutation is the first nonsense mutation in the *PTEN* gene causing exon skipping by disrupting ESE in patients affected with BRRS.

Nonsense mutations can induce the skipping of constitutive exons and one of the possible mechanisms of nonsense-associated altered splicing is the ESE disruption model. A putative role for ESE disruption in nonsense-associated exon skipping has been previously reported in a few disease-causing genes including *DMD*, *BRCA1* and *NF1* (28). Even though nonsense mutations always produce truncated nonfunctional proteins which are deleterious enough to cause diseases regardless of their location with respect to ESEs, it would be interesting to investigate if there is association between reported nonsense mutations in the *PTEN* gene and ESE sites. In addition it has been demonstrated that missense mutations in some cancer predisposition genes, *hMSH2* and *hMLH1* preferentially locate in ESE motifs (22). There is still no report that correlates specific point mutations in *PTEN* coding region with the skipping of the exon harboring the mutation. This prompted us to additionally investigate the possibility of reported missense mutations in the *PTEN* gene being pathogenic because of disrupting ESEs.

We have found that nonsense mutations do not colocalize with ESEs. This result suggests that most of the nonsense-associated exon skipping is not a consequence of ESE disruption. Three other possible mechanisms, e.g. nuclear scanning, indirect nonsense-mediated mRNA decay, or secondary-structure disruption model have been proposed (28). Recent data have also indicated that single-base changes can create negative elements, a splicing silencer (29,30). It is also possible the softwares do not pick up all potential ESE sites. Additional work is needed to better identify the relevant mechanism and machinery responsible for nonsense-associated exon skipping. For analysis of missense mutations, we have also found that missense mutations do not colocalize with ESEs, similar to the studies found in some previously reported diseases, such as metachromatic leukodystrophy (31). This result suggests that pathogenic effects of the majority of missense mutations in the *PTEN* gene are not splicing-related but through other mechanisms, e.g. structural changes and RNA instability. Since *PTEN* encodes a dual-specificity phosphatase which might be sensitive to structural changes, missense mutations causing alterations in amino acids may be as deleterious as those disrupting ESEs.

In summary, we identified a Thai family with Bannayan–Riley–Ruvalcaba syndrome with an additional rare feature, large AVMs in the liver. The molecular analysis revealed an exon skipping-associated nonsense mutation c.511C>T (p.Q171X) in the *PTEN* gene. This mutation has been previously reported in CS, but not in BRRS. The nonsense mutation was predicted to be pathogenic resulting in a truncated protein product. However, we demonstrated here that it also disturbed splicing presumably from disrupting a potential ESE causing skipping of the whole exon 6. This is the first nonsense-mediated exon skipping in the *PTEN* gene being deleterious possibly from disrupting an ESE. The association study between reported pathogenic nonsense/missense mutations and ESE sites, however, revealed that the mutations do not colocalize with ESE sites suggesting that most of their pathogenic effects are through other mechanisms. It would be interesting to investigate the consequence of each *PTEN*

Table 3. ESE motifs found in the *PTEN* gene

SF2/ASF Motifs				SC35 Motifs				SRp40 Motifs				SRp55 Motifs			
Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score	Position in open reading frame		Motif	Score
Left	Right			Left	Right			Left	Right			Left	Right		
50	56	AAGAGGA	3.2	108	115	ATTTCCTG	3.3	2	8	TGACAGC	4.7	114	119	TGCAGA	4.3
136	142	TACAGGA	3.0	133	140	GTATACAG	3.1	35	41	ACAAAAG	3.1	179	184	AGCATA	3.3
287	293	CACAGCT	3.1	325	332	GACCAATG	4.3	135	141	ATACAGG	5.0	247	252	TGCAGA	4.3
384	390	GGGACGA	4.3	364	371	ATTCACTG	3.0	175	181	TCAAAGC	3.9	287	292	CACAGC	3.3
443	449	CACAAGA	4.3	450	457	GGCCCTAG	3.7	192	198	TTACAAG	5.4	357	362	TGCAGC	4.7
511	517	CAGAGGC	3.5	457	464	GATTTCTA	4.1	228	234	TGACACC	4.4	408	413	TGCATA	5.2
586	592	CACAAGA	4.1	477	484	GACCAGAG	3.7	286	292	CCACAGC	5.1	419	424	TACATC	4.9
693	699	CACACGA	6.6	566	573	GACCAGTG	4.7	302	308	TCAAACC	3.6	522	527	TGTGTA	4.2
733	739	CAGCCGT	5.0	615	622	GTTCAGTG	3.8	327	333	CCAATGG	4.2	924	929	TGCAGA	4.3
848	854	CAGAGGA	5.7	649	656	GTCTGCCA	3.6	366	372	TCACTGT	3.7	1036	1041	TACTTC	3.2
999	1005	CAACCGA	3.3	689	696	GACCCACA	3.7	418	424	TTACATC	3.1	1187	1192	AGCATA	3.3
1057	1063	GAGCCGT	3.4	845	852	GACCAGAG	3.7	436	442	TTAAAGG	4.4				
1070	1076	CAGAGGC	3.5	1074	1081	GGCTAGCA	3.7	442	448	GCACAAG	3.3				
1097	1103	CACCAGA	3.1	1086	1093	AACTTCTG	3.3	461	467	TCTATGG	3.5				
1100	1109	CAGATGT	3.5	1143	1150	CACCACTG	3.6	510	516	TCAGAGG	4.6				
1160	1166	CAGAGAA	3.2	1183	1190	GATCAGCA	3.0	568	574	CCAGTGG	4.0				
								585	591	TCACAAG	5.8				
								617	623	TCAGTGG	4.4				
								658	664	CTAAAGG	4.1				
								683	689	ATTCAGG	3.9				
								692	698	CCACACG	5.7				
								705	711	AGACAAG	3.9				
								730	736	CCTCAGC	4.1				
								774	780	CCACAAA	3.0				
								847	853	CCAGAGG	4.2				
								933	939	TGACAAG	4.9				
								1010	1016	TTTCTCC	3.6				
								1040	1046	TCACAAA	3.4				
								1069	1075	CCAGAGG	4.2				
								1140	1146	TGACACC	4.4				
								1145	1151	CCACTGA	3.3				
								1199	1205	TTACAAA	3.1				
								1201	1207	ACAAAAG	3.1				

mutation found using genomic DNA at the transcript level. The differences in the expression level of various transcripts might lead to modification of the phenotype. These findings could have implications to help explain a still unclear genotype-phenotype correlation in the PTEN hamartoma tumor syndrome.

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