รายงานวิจัยฉบับสมบูรณ์

โครงการ

“Molecular Pathogenesis of Rare Craniofacial Anomalies Found in Thailand

โดย

ทันตแพทย์ พีรนิธ กันตะบุตร และคณะ

สิงหาคม 2554

รายงานวิจัยฉบับสมบูรณ์

สัญญาเลขที่ BRG 49800013

"Molecular Pathogenesis of Rare Craniofacial Anomalies Found in Thailand"

1. Principle investigator

ทันตแพทย์ พีรนิธ กันตะบุตร

Division of Pediatric Dentistry,
Department of Orthodontics and Pediatric Dentistry
Faculty of Dentistry, Chiang Mai University
Chiang Mai 50200, THAILAND
Tel: 66-53-944-460; Fax: 66-53-222-844
Email: dentaland17@gmail.com

2. Area of research: Molecular Dysmorphology.

3. Rationales of the Studies:

The main interest of our group is studying the rare congenital craniofacial disorders. Even though they are very rare, they could lead us to the understanding and insight of the genes.

4. Keywords: Tricho-Rhino-Phalangeal syndrome; Neurofibromatosis; Microcephalic Osteodysplastic Primordial Dwarfism; Osteolysis; Beckwith-Wiedemann syndrome; Cleft palate

5. Main specific aims

To search for new syndromes and the genes responsible for those syndrome.

To initiate more international collaborations in the area of molecular genetics and development.

To gain new knowledge in order to improve the quality of life of the Thai patients.

To publish the research in the world renowned journals.

6. Output of TRF-supported Publications since we received this grant

6.1. **Kantaputra P,** Limwongse, Tochareontanaphol C, Mutirangura A, Mevatee U, Praphanphoj V. "New Ectodermal Dysplasia-Holoprosencephaly Syndrome Associated With 45,XY,der(15;18)t(15;18)(q10;q10)" **Am J Med Genet 2006;140A 2598-2602.**

6.2 **Kantaputra PN**, Limwongse C, Koolvisoot A, Ausawamongkolkul A,Tayavitit S. A newly recognized polyosteolysis/hyperostosis syndrome. **Am J Med Genet 2006;140A: 2640-2645**

6.3 Kantaputra P, Miletich I, Lüdecke HJ, Suzuki EY, Praphanphoj V, Shivdasani R, Wuelling M, Vortkamp A, Napierala D, Sharpe PT. Tricho-rhino-phalangeal syndrome with supernumerary teeth. **J Dent Res. 2008; 87:1027-1031.**

6.4 van Haelst MM, Wang R, **Kantaputra PN,** Palmer R, Beales P. Obesity Syndrome, MOMES caused by deletion-duplication (4q35.2 del and 5p15 duplication). **Am J Med Genet A. 2009 Feb 15;149A(4):833-834.**

6.5 Tanpaiboon P, **Kantaputra P,** Wejathikul K, Piyamongkol W. c. 595-596 insC of FOXC2 underlies lymphedema, distichiasis, ptosis, ankyloglossia, and Robin sequence in a Thai patient. **Am J Med Genet A. 2010 Mar;152A(3):737-740.**

6.6 Kantaputra P, Tanpaiboon P, Porntaveetus T, Ohazama A, Sharpe P, Rauch A, Hussadaloy A, Thiel CT. **The smallest teeth in the world are caused by mutations in the *PCNT* gene. Am J Med Genet 2011 155:1398-1403**

6.7 Kantaputra PN, van den Ouweland A Limwonges C. Severe Facial neurofibromatosis. **Lancet** (This paper will be submitted in 2 weeks).

7. **Awards Received**

7.1 Golden Elephant Best Research Award. Health Science Research Award. Chiang Mai University 2006.

7.2 Gordon Research Conference on BONE and TEETH, University of New England, Maine, USA, for Outstanding Poster Presentation on the research entitled “Tricho-Rhino-Phalangeal Syndrome Associated With Multiple Supernumerary Teeth and Mandibular Prognathism” This project is supported by this TRF BRG grant.

7.3 Best Research Award in Oral-maxillofacial Surgery, Oral Pathology & Oral Medicine. IADR Southeast Asian Division 2007, Bali, Indonesia.

8. Dissemination of Knowledge

- Clinical Genetics and Syndromes in Pediatric Dentistry. First Annual Robert J. Feigal Symposium in Pediatric Dentistry. U of Minnesota, October 20, 2007.
- What Face and Teeth Can Tell you about syndromes. University of North Carolina, at Chapel Hill, October 30, 2007
- What Face and Teeth Can Tell you about syndromes. Department of Pediatric Dentistry, University of Iowa, November 5, 2007.

Molecular Dysmorphology of Rare Syndromes. Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan January 8, 2008.

- What Face and Teeth Can Tell you about syndromes. Seminar for Graduate Studies. Tokyo Medical and Dental University. January 11, 2008.
- "Craniofacial Manifestations of *p63*-Associated Ectodermal Dysplasia". EPISTEM Meeting in Ghent, Belgium, February 29, 2008.
- Ectrodactyly, Ectodermal Dysplasia, and Amelogenesis Imperfecta. Gordon Research Conference in Cartilage Biology and Pathology, Switzerland June 2009.
- Phenotypic Analysis of Arg227 Mutations of *TP63* with Emphasis on Dental Phenotype and Micturition Difficulties in EEC Syndrome. Gordon Research Conference in Epithelium: Differentiation and Proliferation. Switzerland June 2009.
- Cleft Lip with Cleft Palate, Ankyloglossia and Hypodontia are Associated with *TBX22* Mutations. Gordon Research Conference in Craniofacial Morphogenesis and Tissue Regeneration. Ill Ilciaco, Lucca, Italy, Arpil, 2010
- *P63* Mutation is associated with Ectrodactyly, Ectodermal Dysplasia, and Amelogenesis Imperfecta. IADR meeting Barcelona, Spain; July 2010
-What Face and teeth can tell you about syndromes. Department of Orthopaedic surgery, National University of Seoul, Korea, November, 2010
-What Face and teeth can tell you about syndromes. Mannipal, India, February 2011
-What Face and teeth can tell you about syndromes. Hyderabad, India, February, 2011
-Oral manifestations of Patients with Mucopolysaccharidosis type VI, Hong Kong, March 2, 2011
- *CLCN7* mutation underlies malignant osteopetrosis in the longest living patient. Poster presentation. Gordon Research Conference: Cartilage Biology and Pathology. Ventura, California, USA March 2011
- Oral manifestations of patients with mucopolysaccharidosis type VI, BioMarin Pharmaceutical Company, California, March 2011
- "The Smallest Teeth in the World are caused by Mutations in *PCNT* gene". Poster and Oral Presentations at Gordon Research Conference: Bones and Teeth. Les Diablerets, Switzerland, June 19-24, 2011.
- Oral manifestations of Patients with Mucopolysaccharidosis type VI. Annual Meetings of The Society of Mucopolysaccharidoses, Poland. July 2011

9. Executive summary

1. We have discovered 2 new genetic disorders (papers 6.1 and 6.2).
2. We found for the first time that the mutation in *TRPS1* gene was responsible for Tricho-Rhino-Phalangeal Syndrome with supernumerary teeth (paper 6.3)
3. We found that 4q35.2 del and 5p15 duplication was the cause of obesity in MOMES syndrome (paper 6.4). We were the first to discover MOMES syndrome (supported by TRF).
4. We found that c.595-596 insC of *FOXC2* gene caused lymphedema, distichiasis, ptosis, ankyloglossia, and Robin sequence (paper 6.5).
5. We were the first who found the etiology of the smallest teeth in the world. It was found in patients with MOPD II (paper 6.6) I was invited to give a presentation of this project a the Gordon Research Conference in Switzerland in June 2011.

10. Progress of the other sub-projects

10.1 DNA has been collected from the family with Beckwith-Wiedemann syndrome and **being sequenced.** (Project 5)

10.2 We have found a large Thai family affected with X-linked hypohidrotic ectodermal dysplasia (โรคสังข์ทอง) in Samut Sakorn Province. Mutation analysis of *EDA* gene of the affected members of this family revealed p.Arg334His and c. 888-905del18 mutations. We are in the process of sequencing this gene in normal controls and patients with non-syndromic missing teeth. This project is the current thesis research of a graduate student in Pediatric Dentistry.

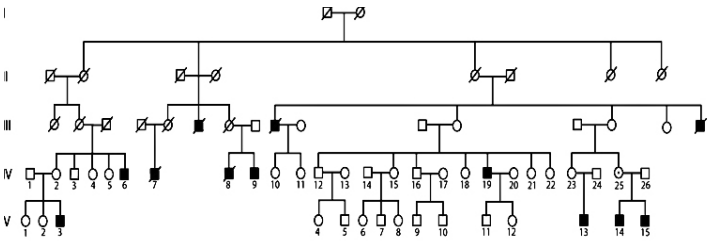
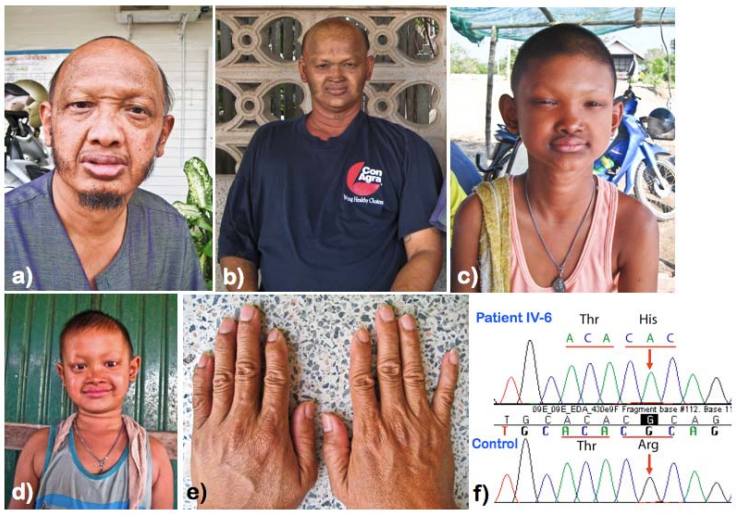
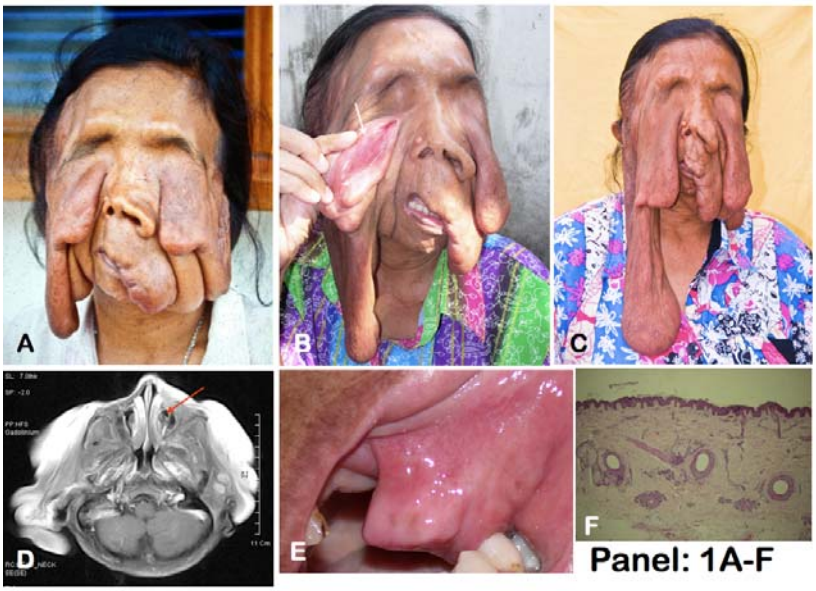


Figure 1. Pedigree of the Thai family affected with XLHED



10.3 Neurofibromatosis

We found a novel mutation (4821delA) in NF1 gene in a Thai woman affected with severe facial manifestation of neurofibromatosis type I. The mutation is predicted to result in truncation of neurofibromin protein. Our manuscript was not accepted by New England Journal of Medicine. We are going to submit to Lancet Journal in 2 weeks.



Panel: 1A-F

11. **Comments:** We have already published 6 international papers from this grant. We are definitely sure we will get at least 3 more after the submission of this final report. Some projects took a long time to find the mutations. That was why this particular BRG grant was a long process

12. **Suggestion:** Thank you for your support. We will end this BRG grant and move the ongoing projects to the other BRG grant of mine (BRG 5280010). The remaining money of this project (BRG 49700013) is expected to be used to pay for the sequencing cost of the ongoing work Krub.

ขอกราบขอบพระคุณ สกว. ที่ให้การสนับสนุนเรามาตลอด มีนักศึกษาปริญญาโทถึง 6 คนที่ใช้เงินจากโครงการนี้ครับ

ทันตแพทย์ พีรนิธ กันตะบุตร

ภาควิชาทันตกรรมสำหรับเด็ก, มหาวิทยาลัยเชียงใหม่

American Journal of Medical Genetics Part A 140A:2598–2602 (2006)

Clinical Report

Contiguous Gene Syndrome of Holoprosencephaly and Hypotrichosis Simplex: Association With an 18p11.3 Deletion

Piranit N. Kantaputra,[1]* Chanin Limwongse,[2] Chintana Tochareontanaphol,[3] Apiwat Mutirangura,[4] Umnat Mevatee,[5] and Verayuth Praphanphoj[6]

[1]Faculty of Dentistry, Department of Pediatric Dentistry, Chiang Mai University, Chiang Mai, Bangkok, Thailand
[2]Faculty of Medicine, Department of Research and Development, Molecular Genetic Unit, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[3]Faculty of Medicine, Research Center, Rama thibodi Hospital, Mahidol University, Bangkok, Thailand
[4]Faculty of Medicine, Division of Genetics, Department of Anatomy, Chulalongkorn University, Bangkok, Thailand
[5]Division of Cytogenetics, Department of Anatomy, Faculty of Medicine, Chiang Mai University, Bangkok, Thailand
[6]Center for Medical Genetics Research, Rajanukul Institute, Bangkok, Thailand

Received 27 March 2006; Accepted 2 June 2006

We report a patient with a unique combination of features, including microcephaly; mental retardation; poorly developed frontal lobes; hypoplastic pituitary gland; hypothyroidism; alopecia universalis; single maxillary central incisor; taurodontism; median palatal ridge; longitudinally grooved nails; and scoliosis. His unbalanced karyotype was found to be 45,XY,der(15;18)(q10;q10). The constellation of anomalies appears to represent a contiguous gene syndrome caused, at least in part, by deletion of *TGIF* and the gene responsible for hereditary hypotrichosis simplex. The phenotype of our patient differs other reported patients with del(18p). Possible explanations include (1) the effects of a different deleted region, (2) a positional effect caused by a gene close by, or (3) by interruption of a different gene resulting from chromosomal translocation.

Key words: poorly developed frontal lobes; mental retardation; hypothyroidism; *TGIF*; single maxillary central incisor; longitudinally grooved nails

How to cite this article: Kantaputra PN, Limwongse C, Tochareontanaphol C, Mutirangura A, Mevatee U, Praphanphoj V. 2006. Contiguous gene syndrome of holoprosencephaly and hypotrichosis simplex: Association with an 18p11.3 deletion. Am J Med Genet Part A 140A:2598–2602.

INTRODUCTION

Here, we report a Thai man with an 18p11.3 deletion. The phenotype consists of a single maxillary central incisor, taurodontism, hypoplastic pituitary gland, mental retardation, alopecia universalis, scoliosis, longitudinally grooved nails, and hypothyroidism. The combination of anomalies found in this patient appears to represent a contiguous gene syndrome.

CLINICAL REPORT

A 27-year-old Thai man was first seen when he was 16 years old. His phenotypic features are shown in Figures 1–3. His facial features are compared at 7, 16, 24, and 27 years of age in Figure 1.

He was the second child of nonconsanguineous parents. His brother and sister were normal. Family history was unremarkable. Birth weight was 2600 g (<10th centile). After birth, he was noted to be hypotonic. At 7 months he was hospitalized for hypothyroidism. He could stand at about 1½ years and walk unassisted at about 2½ years. Speech was delayed. He spoke his first word at 2 years and spoke in sentences at 2½ years. His IQ was estimated to be 50.

*Correspondence to: Piranit N. Kantaputra, Faculty of Dentistry, Department of Pediatric Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand. E-mail: dnpdi001@chiangmai.ac.th
DOI 10.1002/ajmg.a.31386

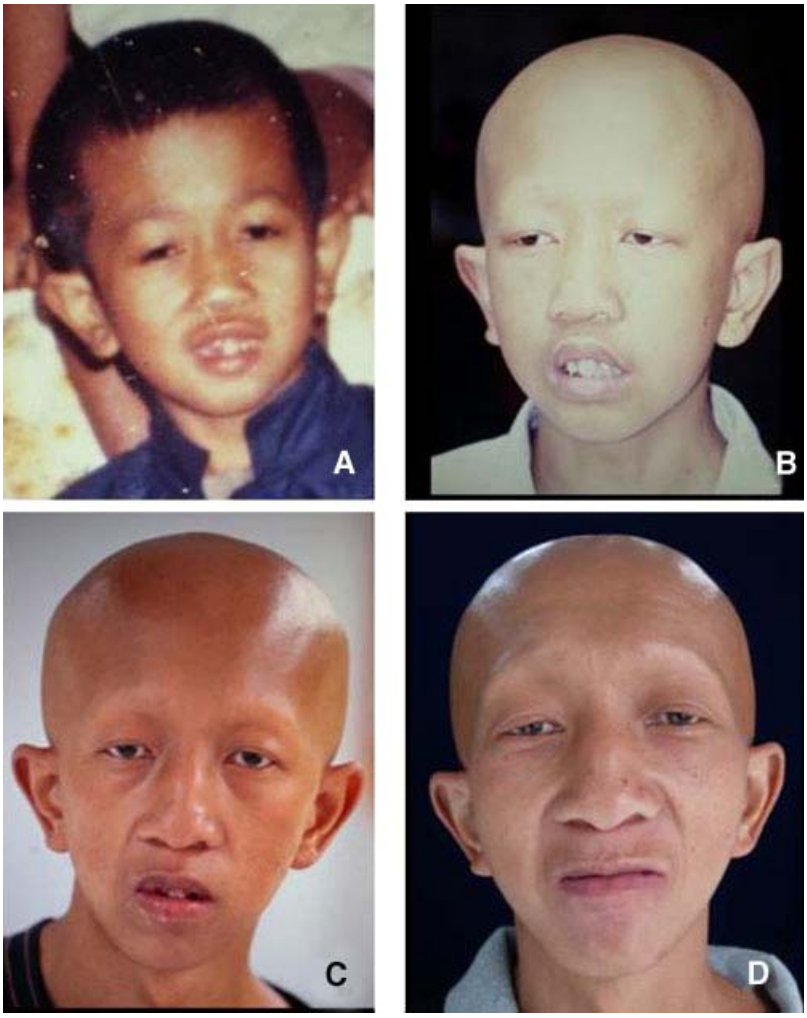


FIG. 1. **A–D**: Patient at 7, 16, 24, and 27 years.

FIG. 2. **A**: Longitudinally grooved thumbnail at 16 years. **B**: The same thumb at 28 years.

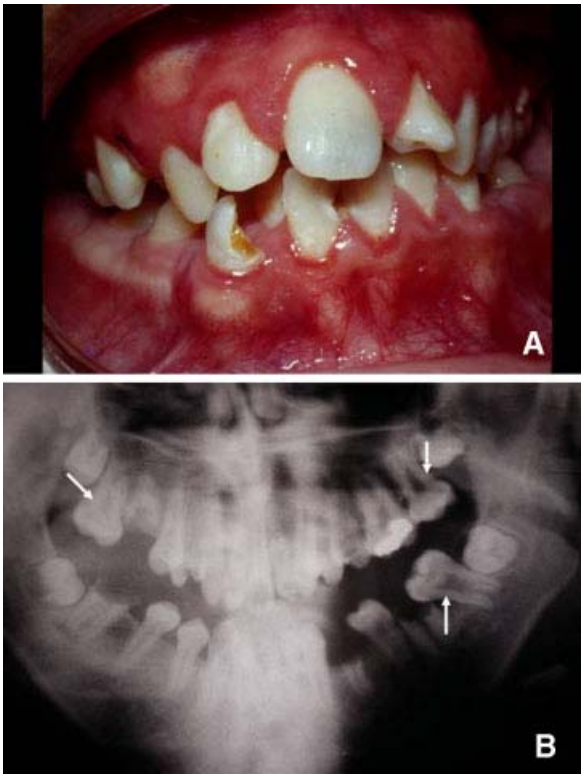


FIG. 3. **A**: Single maxillary central incisor at 16 years. **B**: Taurodontism of molars (arrows).

At 27 years, his weight, height, and OFC were 34 K (<3rd centile), 148 cm (<3rd centile), and 52.5 cm (<3rd centile), respectively. Inter-inner canthal distance was 3 cm (50th centile) and interpupillary distance was 6 cm (75–97th centile) (Fig. 1D). Heights of the father and mother were 170 cm (50th centile) and 150 cm (10–25th centile), respectively. The propositus had normal hair at birth until 6 years when he began to lose it, beginning in the occipital region. By 24 years, he had no scalp hair, eyebrows, eyelashes, nasal hair, body hair, axillary hair, or pubic hair (Fig. 1C). Vellus hair was observed on his cheeks and above his upper lip. A biopsy of the scalp demonstrated the presence of sebaceous and sweat glands but complete absence of hair follicles. His eyebrows and scalp hair in the occipital area started to regrow at 27 years. At 24 years, thyroid function and hearing tests were unremarkable. His fingernails and toenails were thin, brittle, and longitudinally grooved. His fingernails were more severely affected (Fig. 2) than his toenails. His nails improved with age. Oral examination at age 16 years showed a single maxillary central incisor, carious teeth, and a prominent median palatal ridge (Fig. 3A).

Radiographic examination demonstrated a single permanent maxillary central incisor, taurodontism of the permanent molars, multiple carious teeth, and an obtuse mandibular plane angle (Fig. 3B). Cervical spondylosis was demonstrated by spur formation along the anterior margin of C_2–C_5. There was dextroscoliosis of the lower thoracic spine. MRI of the brain disclosed poorly developed frontal lobes, prominent sulci, and small frontal horns of the lateral ventricles. There appeared to be a relative increase of white matter and thinning of gray matter. Myelination was poor. The sella turcica was small (Figs. 4A–D). A posteroanterior radiograph of the skull demonstrated an unusual sinus in the occipital area (Fig. 5).

Chromosomal analysis showed breakage and reunion at 15q10 and 18q10, resulting in monosomy of the short arm of chromosome 18 (Fig. 6). His unbalanced karyotype was 45,XY,der(15;18)(q10;q10). The imprinting analysis using methylation-specific PCR for chromosomes 15 showed appropriate biparental methylation [Kubota et al., 1997].

DISCUSSION

The combination of hair, nail, and dental anomalies found in our patient are all ectodermal components. Alopecia universalis with childhood onset also found in our patient is similar to hereditary hypotrichosis simplex (HTS), which maps to18p11.32-p11.23 [Bentley-Phillips and Grace, 1979; Baumer et al., 2000].

Mental retardation, microcephaly, single maxillary central incisor, prominent median palatal ridge, and structural brain defects can be associated with type 4 holoprosencephaly and has been reported with absence of *TGIF*, which maps to 18p11.3 [Baumer et al., 2000]; del(18p) is one of the most common autosomal deletion syndromes [Schinzel, 2001].

The fact that our patient is affected with both hair loss and holoprosencephaly suggests that his phenotype results from haploinsufficiency of *TGIF* and the gene responsible for HTS; it may be regarded as contiguous gene syndrome. Moreover, other features of his constellation, including transient hypothyroidism, infantile hypotonia, nail defects, tooth abnormalities, and scoliosis should also be caused by haploinsufficiency of genes in this deleted 18p interval.

It should be noted that our patient and patients with HTS had normal hair until 6–8 years. This implies that that the gene for HTS is not necessary for hair growth during childhood, or only a single dose from the other allele is enough for childhood hair growth, but not for adult hair growth. The presence of vellus hair during adulthood implies that it needed less or none of the missing protein and whatever other hair-related proteins he had were enough for vellus growth. Skin biopsy showed absence of scalp hair follicles on histopathlogical examination. Both the disappearance of hair follicles at 24 years and regrowth of hair in the occipital region and eyebrows at 27 years remain unexplained.

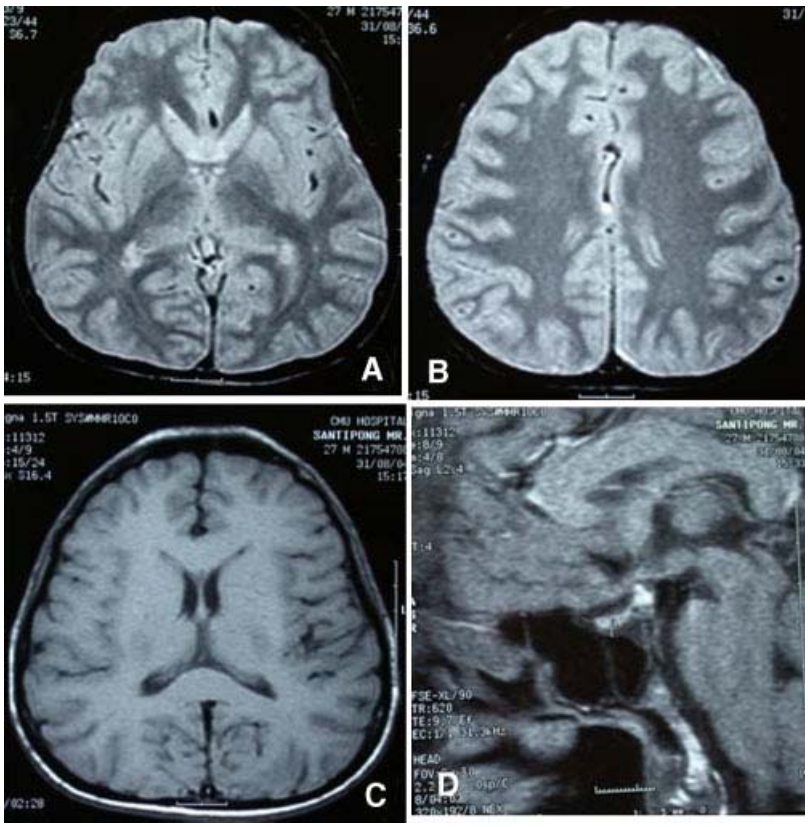


FIG. 4. **A–C**: Poorly developed frontal lobes with deep sulci. Increased white matter and decreased gray mater. **D**: Small sella turcica.

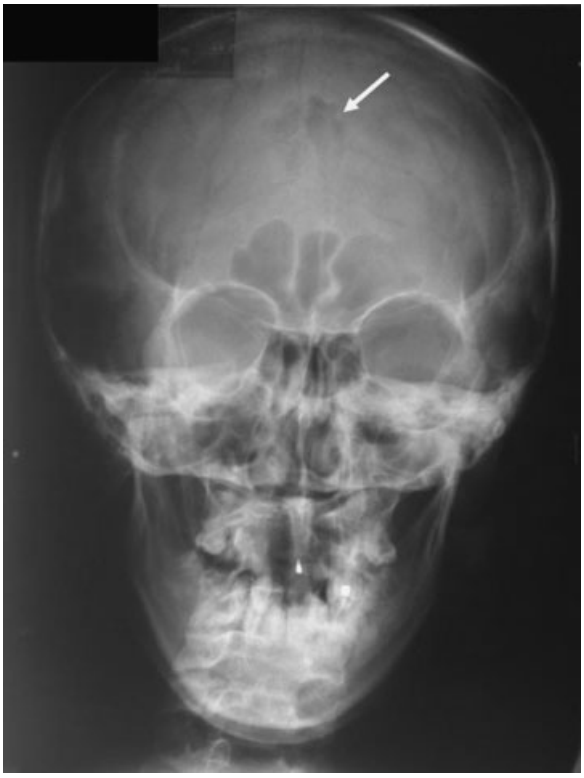


FIG. 5. Unusual sinus in the occipital area (arrow).

Our patient's fingernails were longitudinally grooved at birth. The condition became severe during the teenage years, but improved with age. Apparently, fingernail growth needed less of the missing protein(s) as he aged.

Our patient had multiple carious teeth, which has also been reported in patients with del(18p) [Dolan et al., 1981; Aughton et al., 1991]. Alopecia congenita, holoprosencephaly, and multiple dental caries have

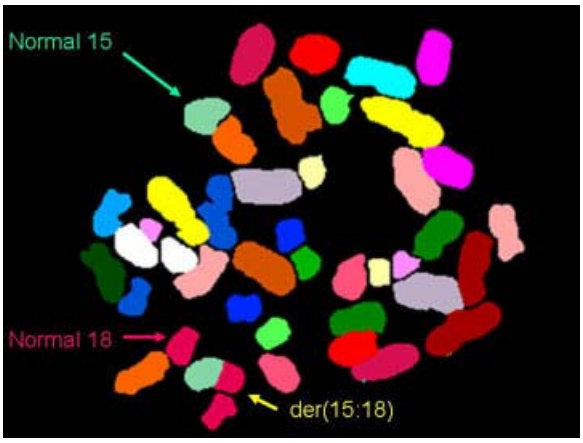


FIG. 6. SKY. Metaphase spread with FISH.

been described in a family with del(18) [Uchida et al., 1965].

The unique combination of anomalies found in our patient appears to represent a contiguous gene syndrome caused, at least in part, by deletion of *TGIF* and the gene responsible for HTS. This was not the effect of uniparental disomy of chromosomes 15 because imprinting analysis showed biparental methylation. The phenotype of our patient differs from other reported patients with del(18p). Possible explanations include (1) the effects of a different deleted region, (2) a positional effect caused by a gene close by, or (3) by interruption of a different gene resulting from chromosomal translocation. Candidate genes in 18p region involved in this constellation of features include *ZFP161* and *LAMA1*.

ACKNOWLEDGMENTS

We thank the patient and his family for allowing us to use their medical and dental information for publication. We thank John A. McGrath for critical review of the manuscript. This research was supported by the Thailand Research Fund (TRF).

REFERENCES

Aughton DJ, AlSaadi AA, Transue J. 1991. Single maxillary incisor in a girl with del(18p) syndrome. J Med Genet 28:530–532.

Baumer A, Belli S, Trueb RM, Schinzel A. 2000. An autosomal dominant form of hereditary hypotrichosis simplex maps to 18p11.32-p11.23 in an Italian family. Eur J Hum Genet 8:443–448.

Bentley-Phillips B, Grace HJ. 1979. Hereditary hypotrichosis. A previously undescribed syndrome. Brit J Dermatol 101:331–339.

Dolan LM, Wilson K, Wilson WG. 1981. 18p- syndrome with single central maxillary incisor. J Med Genet 18:396–398.

Kubota T, Das S, Christian SL, Baylin SB, Herman JG, Ledbetter DH. 1997. Methylation-specific PCR simplifies imprinting analysis. Nat Genet 16:16–17.

Schinzel A. 2001. Catalogue of unbalanced chromosome aberrations in man, 2nd edition. New York: de Gruyter, pp 717–722.

Uchida IA, McRae KN, Wang HC, Ray M. 1965. Familial short arm deficiency of chromosome 18 concomitant with arhinencephaly and alopecia congenital. Am J Hum Genet 17:410–419.

American Journal of Medical Genetics Part A 140A:2640–2645 (2006)

New Syndrome
A Newly Recognized Polyosteolysis/Hyperostosis Syndrome

Piranit N. Kantaputra,[1]* Chanin Limwongse,[2] Ajchara Koolvisoot,[2] Apichart Ausawamongkolkul,[3] and Somsiri Tayavitit[4]

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand
[2]Department of Medicine, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand
[3]Department of Orthopedic Surgery, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand
[4]Department of Radiology, Chumporn Hospital, Chumporn, Thailand

Received 4 May 2006; Accepted 20 May 2006

We report a newly recognized bone disorder consisting of polyostotic expansile osteolysis affecting long bones and iliac bones; hyperostosis of the skull, thoracic cage, and medial portion of both clavicles; pectus carinatum; gigantiform synovial masses of the elbows and knees; atrial septal defect; cardiomegaly; unilateral cryptorchidism; and mental deficiency. Affected bones can be grouped into four general types of skeletal pathology: (1) expansile osteolysis, (2) osteolysis without expansion, (3) expansion without osteolysis, and (4) hyperostosis. Some bones remained unaffected. We have named the condition "polyosteolysis/hyperostosis syndrome." It is clearly at variance with any previously reported bone disorder, including familial expansile osteolysis, juvenile Paget disease, and McCune–Albright syndrome (and polyostotic fibrous dysplasia). Because our patient shared some features in common with juvenile Paget disease, we thought that mutational analysis of *TNFRSF11B* was indicated, even though our patient had some manifestations not found in juvenile Paget disease. Direct sequencing failed to identify a *TNFRSF11B* mutation. Because the parents of our propositus were first cousins suggests that polyosteolysis/hyperostosis syndrome may possibly have autosomal recessive inheritance. © 2006 Wiley-Liss, Inc.

Key words: expansile osteolysis; gigantiform synovial masses; familial Paget disease; osteoprotegerin; *TNFRSF11B*; possible autosomal recessive inheritance

How to cite this article: Kantaputra PN, Limwongse C, Koolvisoot A, Ausawamongkolkul A, Tayavitit S. 2006. A newly recognized polyosteolysis/hyperostosis syndrome. Am J Med Genet Part A 140A:2640–2645.

INTRODUCTION

We report a patient with polyostotic expansile osteolysis affecting the long bones and iliac bones, hyperostosis of the skull and thoracic cage, pectus carinatum, gigantiform synovial masses of the elbows and knees, ASD, cardiomegaly, cryptorchidism, and mental retardation. To our knowledge, this constellation of findings has not been described previously; it may represent a newly recognized autosomal recessive syndrome.

CLINICAL REPORT

An 11-year-old Thai boy was first seen for multiple joint swellings. He was the oldest of four children from a first cousin marriage. Two younger brothers and sister were normal (Fig. 1a). Another sister was deceased from an unrelated cause.

Ten days after birth, his elbows and knees began to swell and became larger with age. At 7 months, he was noted to be cyanotic when crying. Clubbing of the fingers and toes was observed. A small ASD and cardiomegaly were detected at 3 years. At 4 years, his mother noted that he tired easily. Hepatomegaly was detected at that time. He became anemic and occasionally required a blood transfusion. Limited extension of his fingers was observed. He fell and broke his left arm at 9 years. At 11 years, the swollen elbows and knees became greatly enlarged. His

Grant sponsor: Thailand Research Fund (TRF); Grant sponsor: Mahidol University Research Fund.
*Correspondence to: Piranit N. Kantaputra, Department of Pediatric Dentistry, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200, Thailand. E-mail: dnpdi001@chiangmai.ac.th
DOI 10.1002/ajmg.a.31373

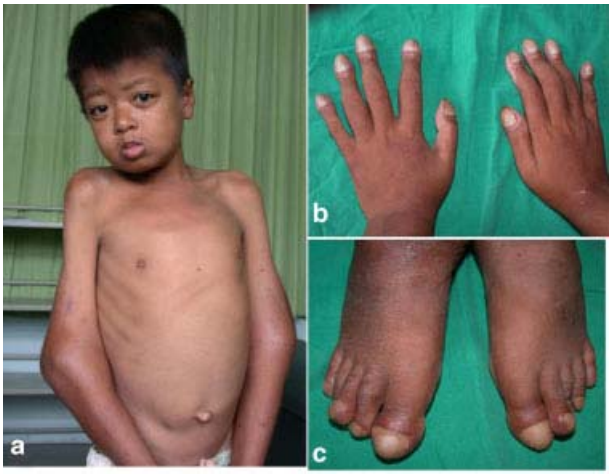


FIG. 1. (**a**) Patient at 12 years. Note yellowish sclerae, swollen elbows, wasting of the upper arm muscles, hyperpigmentation of the skin at the middle of the arm, pectus carinatum, protruding abdomen, and umbilical hernia. (**b**) Clubbing of fingers and thumbs with flexion contracture. (**c**) Clubbing of toes.

parents noted that, at times, his periarticular regions were warmer than other parts of his body.

At 12 years, his weight, height, and OFC were 30.5 kg (25th–50th centile), 128 cm (3rd centile), and 49 cm (3rd centile), respectively. His cheeks were full and his tongue was pale. Cervical and submandibular lymphadenopathy was noted and was most likely due to multiple dental abscesses and chronic periodontitis. Protruding abdomen with hepatoplenomegaly and umbilical hernia were observed (Fig. 1a). The left testis was palpable in the inguinal canal. His right testis was within the scrotal sac and was 1 cm in diameter. Stretched penile length was 4.5 cm. Pubic hair was not observed. His elbows and knees were symmetrically swollen with a soft cystic consistency (left elbow, 31 cm in diameter; right elbow, 27 cm in diameter; left knee, 41 cm in diameter; right knee, 40 cm in diameter) (Figs. 1a and 2a). The skin over the swellings was warm and had finely dilated superficial veins. Mild limitation of flexion and extension of the fingers, elbows, and knees was observed. His ankles were swollen medially. His fingers and toes exhibited clubbing (Fig. 1b,c). Wasting of the upper muscles of the upper arms was noted (Fig. 1a).

Developmental Assessment

Developmental evaluation at birth appeared to be normal. He sat, stood, and walked at 6 months, 1 year, and 2 years, respectively. After 2 years, his parents noted gradual developmental delay. He spoke his first word at 2 years. At 7 years, he attended school for 2–3 months and then stopped because of motor difficulty. At 12 years, mental development was severely delayed with an IQ of 24 (Stanford-Binet). He appeared to have more advanced receptive language compared to his minimally expressive language skills.

Laboratory Examination

The patient had chronic hypochromic, microcytic anemia. Hemoglobin electrophoresis yielded a normal hemoglobin type. Other findings included mild elevation of serum phosphorus (5.9 mg/dl: norm 2.2–5.0 mg/dl), but age-appropriate levels of calcium (8.1 mg/dl; norm 8.1–10.4 mg/dl), alkaline phosphatase (151 U/L; norm 39–117 U/L), and parathyroid hormone. Urinalysis, liver function tests, serum BUN (7 mg/dl: norm 5–25 mg/dl), and creatinine (0.4 mg/dl: norm 0.5–1.5 mg/dl) were normal. Elevated levels of beta crosslap (1.56 ng/ml: norm 0.00–0.32) and osteocalcin (196.80 ng/ml: norm 1.00–35.00 ng/ml) indicated increased osteoclastic activity. Synovial tapping of the left knee yielded 150 ml of a clear yellowish joint fluid. Joint fluid studies disclosed a glucose of 95 mg/dl and a very low number of white blood cells, consistent with a non-inflammatory cause of the swelling.

Synovial Pathology

The patient underwent total synovectomy of the left knee at 13 years. The external surface of the synovium appeared as white fibrous tissue with areas of fatty tissue. The internal surface had a smooth and shiny grayish-white surface. The synovial mass contained numerous large yellowish villi (Fig. 2b). The cut surface showed rubbery, grayish-white homogeneous tissue. After removing the synovium, the distal femoral cartilage appeared erosive. Histological study of the synovium disclosed non-specific chronic synovitis with fatty infiltration (Fig. 2c). Bone marrow biopsy at the distal femur showed areas of

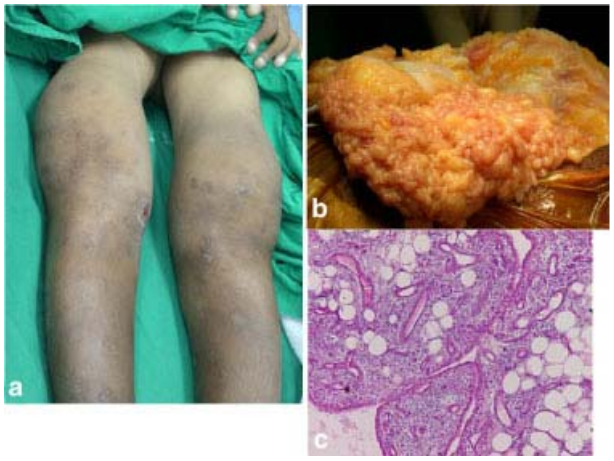


FIG. 2. (**a**) Swollen knees with hyperpigmented skin. (**b**) Gigantiform synovial mass of hyperplastic villi of the knee joint. (**c**) Histopathology showing nonspecific chronic synovitis with fatty infiltration.

hyper- and hypo-cellularity and adequate numbers of precursor cells; there was no evidence of a tumor or a granuloma. Two years after total synovectomy, the knee joint was reported to have enlarged to the same size as noted previously.

Radiographic Findings

Affected bones grouped according to skeletal pathology are listed in Table I. Radiographic findings of the skull included thick cranial tables with a widened medullary space and a hair-on-end appearance, hyperostotic cranial vault, and hyperostotic cranial base. The posterior cranial base was flat (Figs. 3a,b). A panoramic radiograph disclosed an underdeveloped mandible, multiple dental caries, and taurodontism of the maxillary second permanent molars. There were multiple areas of unusual and severe bone loss resulting from dental infection (Fig. 3c). Chest radiograph showed a hyperostotic thoracic cage and broad ribs (Fig. 4b). The medial portion of each clavicle showed a thick cortex and narrow medullary space. The lateral portion of each clavicle appeared small. Cardiomegaly was present. The vertebrae were unremarkable (Figs. 4b and 5b).

Diaphyseal/metaphyseal expansion was somewhat symmetric with multiple osteolytic lesions and very thin cortices. Expansile osteolytic lesions were present at the distal ends of the humeri and proximal ends of the radii and ulnae; all had extremely thin cortices. Trabecular septae had an irregular pattern (Fig. 6a,b). The distal radii, ulnae, and carpal bones were unremarkable except for thin cortices. Large soft tissue masses were evident at the elbows (Figs. 6a,b). Radiographs of the hands at 11 and 12 years showed medially dislocated distal phalanges of both thumbs and narrow proximal interphalangeal joint spaces of digits 2–4. The middle phalanges of fingers 2–5 did not appear to have broad ends. Progressive acro-osteolysis was observed in the distal phalanges of the thumbs and fingers 2–5. The distal phalanx of fingers 5 on both sides appeared tapered (Fig. 4a).

A pelvic radiograph showed multiple large osteolytic lesions of the iliac bones particularly the iliac wings. The cortices appeared very thin. Expansion of iliac bones was not observed. The pubic bones, ischium, and patella were unremarkable. The proximal femoral diaphyses were severely affected by large expansile osteolytic lesions. The cortical bones were extremely thin (Figs. 4d and 7a,b). The diaphyses and metaphyses of the tibiae and fibulae appeared expanded but without evidence of osteolytic lesions. Multiple, opaque transverse lines were noted in the middle portions of the tibiae and fibulae. (Figs. 4d and 5a). An MRI of the knees disclosed large soft tissue masses occupying the synovial cavities of the joints. Some areas of the mass had a low signal, which was consistent with fluid content. There were infiltrative lesions in the medullary cavities of the long bones, including the tibiae, fibulae, and femora

TABLE I. Skeletal Findings

Expansile osteolysis
Distal humerus
Proximal radius
Proximal ulna
Distal phalanges of digits
Proximal femur
Expansion without osteolysis
Distal radius
Distal ulna
Distal femur
Tibia
Fibula
Osteolysis without expansion
Lateral clavicle
Iliac bone
Hyperostosis
Cranial base
Thoracic cage and ribs
Medial clavicle
Unaffected bones
Proximal humerus
Carpal bones
Vertebrae
Proximal end of femur
Tarsal bones
Pubic bone
Ischium

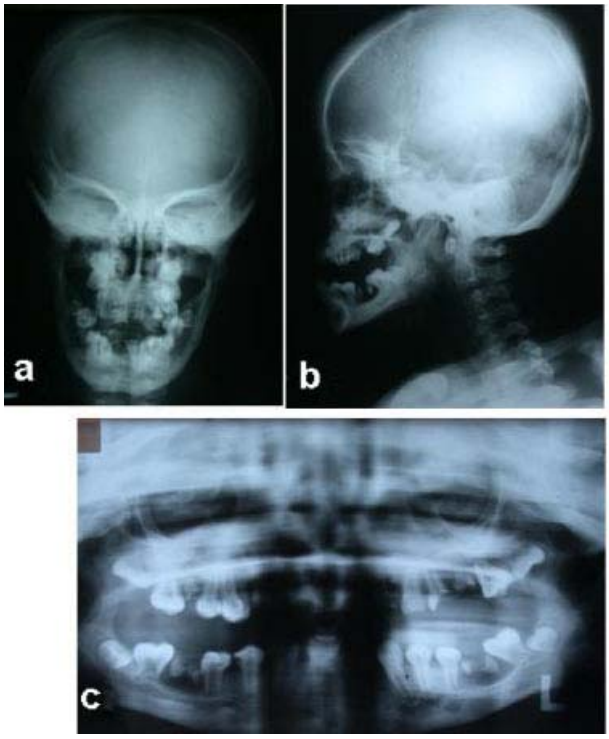


FIG. 3. (**a**) PA skull radiograph and (**b**) lateral cephalograph showing widened diploe and hyperostosis of the cranial base. The outer and inner tables of the cranium are thin. (**c**) Panoramic radiograph showing taurodontism of maxillary second molars and severe alveolar bone loss caused by dental infection.

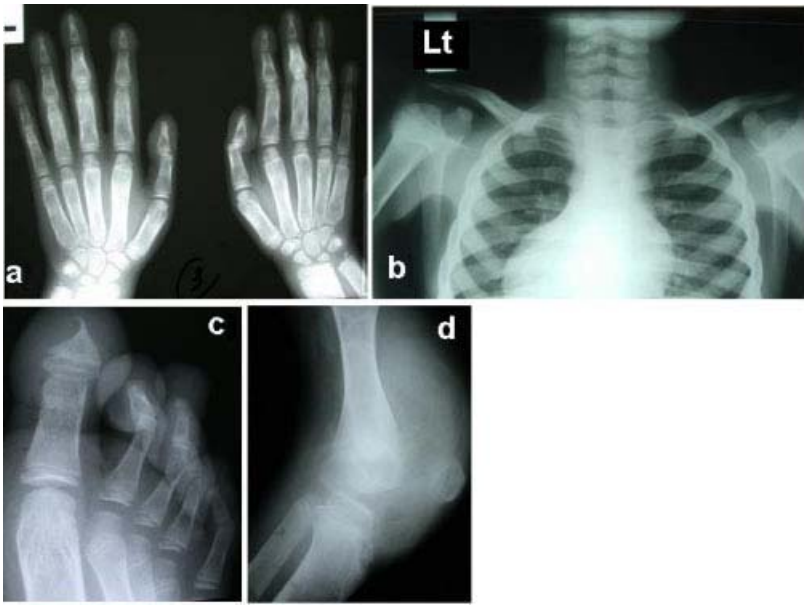


FIG. 4. (**a**) Radiograph showing narrow proximal interphalangeal spaces of digits 2–4. Acro-osteolysis of distal phalanges. (**b**) Radiograph showing hyperostotic thoracic cage with broad ribs. The lateral portions of both clavicles are hypoplastic. Cardiomegaly is noted. (**c**) Severe acro-osteolysis of toes. (**d**) Expanded metaphyses of the distal femur, proximal tibia, and fibula. The patella is unremarkable.

with expansion of the metaphyses and diaphyses (Fig. 8). Acro-osteolysis was severe in the distal phalanges of the toes. Distal symphalangism was noted on the left fifth toe. The tarsal bones were unremarkable (Fig. 4c).

Mutational Analysis of *TNFRSF11B*

TNFRSF11B encodes osteoprotegerin (OPG), a member of the tumor necrosis factor (TNF) receptor superfamily, and which acts as a decoy receptor that can bind to RANKL. A high frequency of OPG favors increased bone mass, whereas a low frequency of OPG favors bone resorption. Mutations in *TNFRS11B* are known to cause juvenile Paget disease [Cundy et al., 2002, 2005; Whyte et al., 2002a; Chong et al., 2003; Teitelbaum and Ross, 2003; Janssens et al., 2005]. Because our patient shared some features in common with juvenile Paget disease, such as thickened cranium, polyostotic osteolytic lesions,

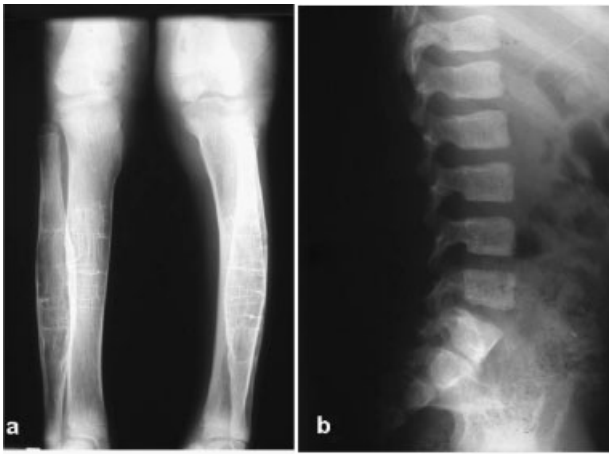


FIG. 5. (**a**) Enlarged tibiae and fibulae. The left tibia is slightly bowed. Multiple opaque transverse lines are seen in the middle portions of the tibiae. (**b**) The vertebrae are unremarkable.

FIG. 6. Radiographs (**a**) right and (**b**) left arms, (**c**) right and (**d**) left elbows. Enlargement of the distal humerus, and proximal radius and ulna with multiple expansile osteolytic lesions. Large soft tissue mass at the elbow. Cortex is thin and trabecular septae irregular.

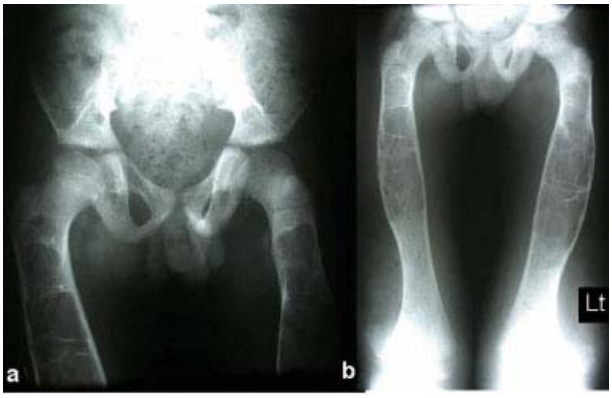


FIG. 7. (**a**) Pelvic radiograph showing multiple large osteolytic lesions of the iliac bones and proximal femora. Cortical bones are extremely thin. (**b**) Note osteolysis of the proximal femora. Proximal tibiae and fibulae and distal femora are large with no evidence of osteolytic lesions.

ing long bones and iliac bones; hyperostosis of the skull, thoracic cage, and medial portion of the clavicle; pectus carinatum; gigantiform synovial masses of the elbows and knees; ASD; cardiomegaly; cryptorchidism; and mental retardation. Affected bones grouped according to skeletal pathology are listed in Table I. General groupings include (1) expansile osteolysis, (2) expansion without osteolysis, (3) osteolysis without expansion, and (4) hyperostosis. Unaffected bones (5) are also listed in Table I.

We were unable to obtain a bone biopsy from either an expansile osteolytic lesion or a hyperostotic lesion. The gigantiform synovial lesion was diagnosed as benign synovial villous hypertrophy. Such lesions occurred very soon after birth and gradually enlarged, causing joint effusion and a limited range

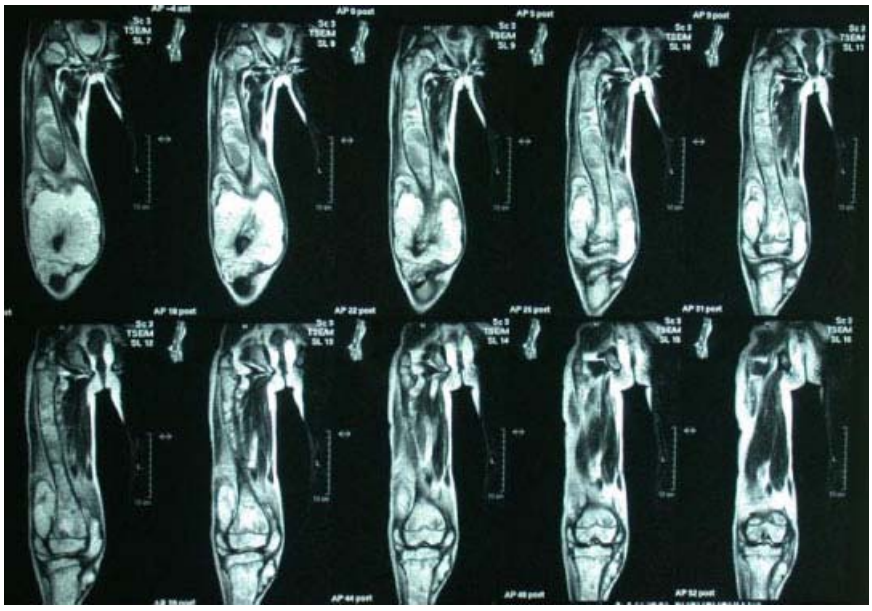


FIG. 8. Infiltrative lesions in the medullary cavities of the long bones, including the tibiae, fibulae, and femora with expansion of the metaphyses and diaphyses.

thin cortical bones, and expansion of long bones, we thought that mutational analysis of *TNFRSF11B* was indicated even though our patient had some manifestations not found in juvenile Paget disease.

Genomic DNA was isolated from peripheral blood samples. Amplification of the entire coding sequence was performed using primer sequences and PCR conditions described elsewhere [Cundy et al., 2002]. Direct sequencing did not identify a *TNFRSF11B* mutation.

DISCUSSION

We have reported an intriguing bone disorder consisting of polyostotic expansile osteolysis affect-

of motion at the elbows and knees because of chronic synovitis.

To our knowledge, this represents a newly recognized polyosteolysis/hyperostosis syndrome that is at variance with any previously reported bone disorder, including familial expansile osteolysis [Hughes et al., 2000; Palenzuela et al., 2002; OMIM, 2006], juvenile Paget disease [Golob et al., 1996; Whyte et al., 2002a; OMIM, 2006], and McCune–Albright syndrome (and polyostotic fibrous dysplasia) [Cohen and Howell, 1999; de Sanctis et al., 1999; OMIM, 2006].

The parents were first cousins, suggesting that this polyosteolysis/hyperostosis syndrome may have autosomal recessive inheritance.

ACKNOWLEDGMENTS

We are grateful to the patient and his family for their participation, generosity, kindness, and especially patience. We thank Suchart Benjarasmeeroj, Department of Pathology, Faculty of Medicine Siriraj Hospital for histopathological study of the synovium, and Somyod Tayavitit who introduced the patient to us. This work is dedicated to the patient and his family.

REFERENCES

Chong B, Hegde M, Fawkner M, Simonet S, Cassinelli H, Coker M, Kanis J, Seidel J, Tau C, Tuysuz B, Yuksel B, Love D, International Hyperphosphatasia Collaborative Group. 2003. Idiopathic hyperphosphatasia and *TNFRSF11B* mutations: Relationships between phenotype and genotype. J Bone Miner Res 18:2095–2104.

Cohen MM Jr, Howell RE. 1999. Etiology of fibrous dysplasia and McCune-Albright syndrome. Int J Oral Maxillofac Surg 28:366–371.

Cundy T, Hegde M, Naot D, Chong B, King A, Wallace R, Mulley J, Love DR, Seidel J, Fawkner M, Banovic T, Callon KE, Grey AB, Reid IR, Middleton-Hardie CA, Cornish J. 2002. A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. Hum Mol Genet 11:2119–2127.

Cundy T, Davidson J, Rutland MD, Stewart C, DePaoli AM. 2005. Recombinant osteoprotegerin for juvenile Paget's disease. N Engl J Med 353:918–923.

de Sanctis C, Lala R, Matarazzo P, Balsamo A, Bergamaschi R, Cappa M, Cisternino M, de Sanctis V, Lucci M, Franzese A, Ghizzoni L, Pasquino AM, Segni M, Rigon F, Saggese G, Bertelloni S, Buzi F. 1999. McCune-Albright syndrome: A longitudinal clinical study of 32 patients. J Pediatr Endocrinol Metab 12:817–826.

Golob DS, McAlister WH, Mills BG, Fedde KN, Reinus WR, Teitelbaum SL, Beeki S, Whyte MP. 1996. Juvenile Paget disease: Life-long features of a mildly affected young woman. J Bone Miner Res 11:132–142.

Hughes AE, Ralston SH, Marken J, Bell C, MacPherson H, Wallace RG, van Hul W, Whyte MP, Nakatsuka K, Hovy L, Anderson DM. 2000. Mutations in *TNFRSF11A*, affecting the signal peptide of RANK, cause familial expansile osteolysis. Nat Genet 24:45–48.

Janssens K, de Vernejoul MC, de Freitas F, Vanhoenacker F, Van Hul W. 2005. An intermediate form of juvenile Paget's disease caused by a truncating TNFRSF11B mutation. Bone 36:542–548.

OMIM. 2006. Online Mendelian Inheritance in Man.

Palenzuela L, Vives-Bauza C, Fernandez-Cadenas I, Meseguer A, Font N, Sarret E, Schwartz S, Andreu AL. 2002. Familial expansile osteolysis in a large Spanish kindred resulting from an insertion mutation in the *TNFRSF11A* gene. J Med Genet 39:E67.

Teitelbaum SL, Ross FP. 2003. Genetic regulation of osteoclast development and function. Nat Rev Genet 4:638–649.

Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH, Mumm S. 2002a. Osteoprotegerin deficiency and juvenile Paget's disease. N Engl J Med 18 347:175–184.

RESEARCH REPORTS
Clinical

P. Kantaputra[1]*, I. Miletich[2]*, H.-J. Lüdecke[3], E.Y. Suzuki[4], V. Praphanphoj[5], R. Shivdasani[6], M. Wuelling[7], A. Vortkamp[7], D. Napierala[8], and P.T. Sharpe[2]+

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Chiang Mai University, Thailand; [2]Department of Craniofacial Development, Dental Institute, Biomedical Research Centre, King's College London, United Kingdom; [3]Institut fur Humangenetik, Universitätsklinikum, Essen, Germany; [4]Department of Orthodontics, Faculty of Dentistry, Chiang Mai University, Thailand, [5]Genetic Laboratory Rajanukul Institute, Bangkok, Thailand, [6]Dana-Farber Cancer Institute, Boston, MA, USA, [7]Department of Developmental Biology, Center for Medical Biotechnology, University Duisburg-Essen, Essen, Germany; and [8]Baylor College of Medicine, Houston, TX, USA; *authors contributing equally; +corresponding author, paul.sharpe@kcl.ac.uk

J Dent Res 87(11):1027-1031, 2008

ABSTRACT

Tricho-rhino-phalangeal syndromes (TRPS) are caused by mutation or deletion of *TRPS1*, a gene encoding a GATA transcription factor. These disorders are characterized by abnormalities of the hair, face, and selected bones. Rare cases of individuals with TRPS displaying supernumerary teeth have been reported, but none of these has been examined molecularly. We used two different approaches to investigate a possible role of TRPS1 during tooth development. We looked at the expression of *Tprs1* during mouse tooth development and analyzed the craniofacial defects of *Trps1* mutant mice. In parallel, we investigated whether a 17-year-old Thai boy with clinical features of TRPS and 5 supernumerary teeth had mutation in *TRPS1*. We report here that *Trps1* is expressed during mouse tooth development, and that an individual with TRPS with supernumerary teeth has the amino acid substitution A919V in the GATA zinc finger of *TRPS1*. These results suggest a role for *TRPS1* in tooth morphogenesis.

KEY WORDS: Tricho-rhino-phalangeal syndrome, TRPS1, tooth development, supernumerary teeth.

Received April 23, 2007; Last revision May 29, 2008; Accepted August 1, 2008

Tricho-Rhino-Phalangeal Syndrome with Supernumerary Teeth

INTRODUCTION

The Tricho-Rhino-Phalangeal Syndromes (TRPS), first described by Giedion in 1966, are a group of human autosomal-dominant developmental disorders characterized by abnormalities of the hair, face, and selected bones. Individuals with TRPS have sparse fine and slow-growing scalp hair, laterally sparse eyebrows, sparse eyelashes, a bulbous tip of the nose, a large and flat filtrum, a thin upper lip, occasionally large and protruding ears, cone-shaped epiphyses of phalangeal bones, and hip malformations (Giedion, 1966; Giedion *et al.* 1973).

The TRPS have been classified into three types according to their clinical phenotypes and cytogenetic abnormalities. TRPS type I, or Giedion syndrome (TRPS I; OMIM #190350), the prototype of this group of disorders, is caused by deletion or heterozygous mutations in the *TRPS1* gene on chromosome 8q24.12 (Momeni *et al.*, 2000; Lüdecke *et al.*, 2001). *TRPS1* encodes a large nuclear protein with 9 predicted zinc finger (Zfn) domains, including one GATA-type Znf and a carboxy-terminal IKAROS-like double Zfn. Contrary to other GATA transcription factors that activate transcription, TRPS1 behaves as a strong transcriptional repressor both *in vitro* and *in vivo* (Malik *et al.*, 2001, 2002).

TRPS II, or Langer-Giegion syndrome (OMIM #150230), is a contiguous gene syndrome and is the result of microdeletion of the contiguous *TRPS1* and *EXT1* genes. In addition to the TRPS I abnormalities, persons affected with TRPS II exhibit multiple exostoses, predominantly at the ends of the long bones, and some persons with very large deletions tend to have mental retardation (Bühler *et al.*, 1980, 1987; Langer *et al.*, 1984; Yamamoto *et al.*, 1989; Lüdecke *et al.*, 1995, 1999).

TRPS III, or Sugio-Kajii syndrome (OMIM #190351), is a form of TRPS with severe short stature and severe brachydactyly as a result of short metacarpals, but without any exostoses, and can be considered a severe form of TRPS I, but not a distinct entity (Niikawa and Kamei, 1986; Lüdecke *et al.*, 2001).

In addition to TRPS' characteristic abnormalities, occasional dental-associated phenotypes have been described, such as microdontia (Goodman *et al.*, 1981), delayed tooth eruption (Hussels, 1971), and malocclusion (Hussels, 1971; Pashayan *et al.*, 1974; Howell and Wynne-Davies, 1986). Interestingly, supernumerary teeth have been observed in several persons with TRPS (for review, see Gorlin *et al.*, 2001). For example, the originally reported individual (Giedion, 1966) had 2 supernumerary incisors. However, the presence of mutations in the *TRPS1* gene was not investigated in TRPS associated with supernumerary teeth.

To investigate a possible role of *TRPS1* during tooth development, we used two different approaches. We used mouse embryos to study *Trps1* expression during tooth development and took advantage of *Trps1*$^{\Delta gt}$ mutant mice (Malik *et al.*, 2002) to analyze the orofacial phenotype of mice lacking the GATA domain of *Trps1*. In parallel, we looked for a *TRPS1* mutation in a person presenting clinical features of TRPS with supernumerary teeth and mandibular prognathism.

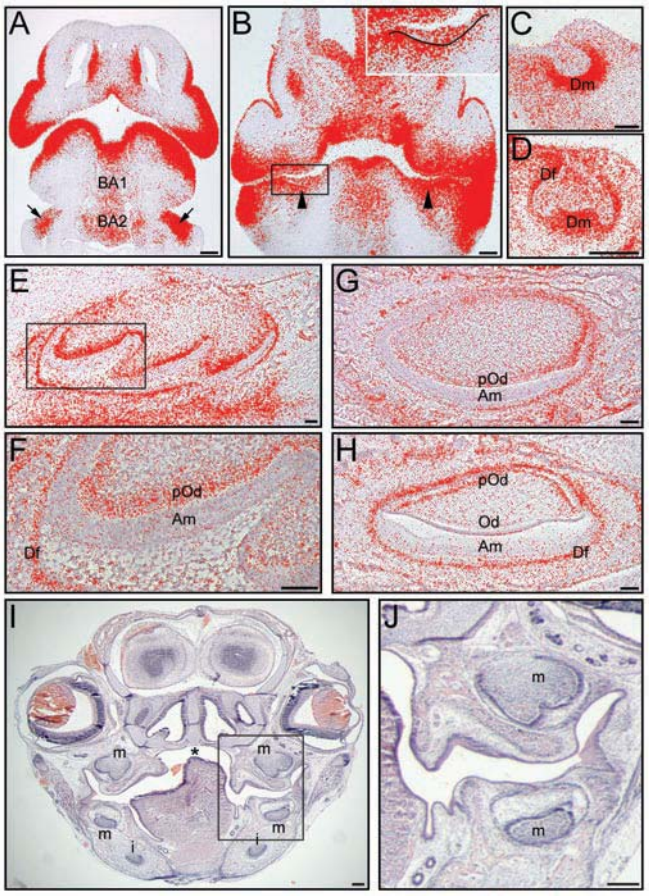


Figure 1. Expression of *Trps1* during mouse tooth development and craniofacial defects observed in *Trps1*$^{-/-}$ mice. *In situ* hybridization for Trps1 on coronal **(A-D)** and sagittal **(E-H)** sections of E11.5 **(A,B)**, E13.5 **(C)**, E14.5 **(D)**, and P0 **(E-H)** first lower molar **(B-D)** first upper molar **(E,F)**, and lower incisor **(G,H)** tooth germs. **(A)** In the first branchial arch, *Trps1* expression is restricted to the oral mesenchyme. Note *Trps1* expression in the second branchial arch at the level of the cleft (arrows). **(B)** Molar epithelial thickenings are indicated with arrowheads. The area boxed in white is a higher magnification of the area boxed in black, with the molar epithelial thickening outlined in black. **(F)** A higher magnification of the area boxed in (E). **(I,J)** Coronal sections of an E18.5 *Trps1*$^{\Delta gt}$/*Trps1*$^{\Delta gt}$ head stained with hematoxylin and eosin. (I) Cleft palate is indicated with a star. A correct number of molar and incisor tooth germs was observed. (J) Higher magnification of the area boxed in (I), showing upper and lower molar tooth germs with a normal shape. Am, ameloblasts; BA1, first branchial arch; BA2, second branchial arch; Df, dental follicle; Dm, dental mesenchyme; i, incisors; m, molars; Od, odontoblasts; pOd, pre-odontoblasts. Scale bar = 100 μm.

MATERIALS & METHODS

In situ Hybridization

Wild-type mouse embryos were collected from CD-1 mice. Radioactive *in situ* hybridization with ^{35}S-UTP labeled RNA probes was carried out as previously described (Wilkinson, 1992) on paraffin tissue sections. *Trps1* antisense riboprobe was generated as in Miletich *et al.* (2005) from a mouse cDNA clone containing a full-length *Trps1* cDNA. Slides were counterstained with hematoxylin (Fluka, Poole, Dorset, UK). All animal experiments were carried out in accordance with UK Home Office regulations.

Analysis of *Trps1*$^{\Delta gt}$/*Trps1*$^{\Delta gt}$ Mice

Homozygous mice with deletion of the TRPS1 GATA domain have been previously described (Malik *et al.*, 2002). Frontal sections were stained with hematoxylin and eosin.

Mutation Analysis

The individual participated after providing informed consent to a protocol that was reviewed and approved by the Institutional Review Board of Chiang Mai University. Following informed consent, given by his mother, high-molecular-weight DNA was extracted from the participant from peripheral leukocytes with the use of the NUCLEON II kit (Amersham). Individual exons of the *TRPS1* gene were amplified by polymerase chain-reaction (PCR) and sequenced, as described previously (Momeni *et al.*, 2000; Lüdecke *et al.*, 2001). Specifically, to amplify exon 6 and flanking introns of *TRPS1*, we used the following primers: forward primer 5′-catgtgactcacctctgacct-3′ and reverse primer 5′-aactacaaggcggttgtcatcag-3′. The expected PCR product had 315 base pairs (bp).

RESULTS

Trps1 is Expressed during Mouse Tooth Development

Trps1 expression has previously been found in many developing organs of the mouse (Kunath *et al.*, 2002). This study reported early facial expression of *Trps1* in the pharyngeal arches, and later expression in the developing muscles of the tongue and surrounding most of the developing cartilage anlagen of the facial bones. To investigate a possible function of *Trps1* during tooth development, we analyzed *Trps1* expression from tooth initiation at embryonic day 11.5 (E11.5) to an advanced stage of crown morphogenesis at post-natal day 0 (P0). At E11.5, when localized thickenings of the oral epithelium mark the future sites of tooth development, *Trps1* is expressed in both the epithelial thickenings and the underlying neural-crest-derived mesenchyme (Fig. 1B). In the first branchial arch, which will subsequently give rise to the lower jaw, *Trps1* mesenchymal expression appears restricted to the oral part of the mandible (Fig. 1A). Subsequently, at the bud (E13.5) and cap (E14.5) stages, *Trps1* is strongly expressed in the condensing dental mesenchyme and the future dental follicle (Figs. 1C, 1D). At birth, crown morphogenesis is almost complete, and two specialized cell types—the ameloblasts and odontoblasts—have differentiated, and are responsible for the secretion of enamel and dentin matrices, respectively. At this stage, *Trps1* is strongly up-regulated in non-secreting pre-odontoblasts in both molars and incisors (Figs. 1E-1G). *Trps1* expression is then down-regulated as soon as pre-odontoblasts fully differentiate in mature odontoblasts and start secreting dentin matrix (Fig. 1H). Strong expression of *Trps1* is maintained in the dental follicle (Figs. 1E-1H).

To identify a functional role for *Trps1* during tooth development, we analyzed mice carrying a deletion of the GATA Zfn domain of *Trps1* (Malik *et al.*, 2002). E18.5 mouse embryos homozygous mutant for *Trps1* had no obvious dental abnormalities. They exhibited the expected number of teeth (3 molars and 1 incisor *per* quadrant) (Fig. 1I), and the shapes of molar and incisor crowns appeared to be normal (Fig. 1J). No ectopic budding of the oral epithelium that would be suggestive of the arrested development of supernumerary teeth was observed at earlier stages of tooth development in *Trps1*$^{\Delta gt}$/

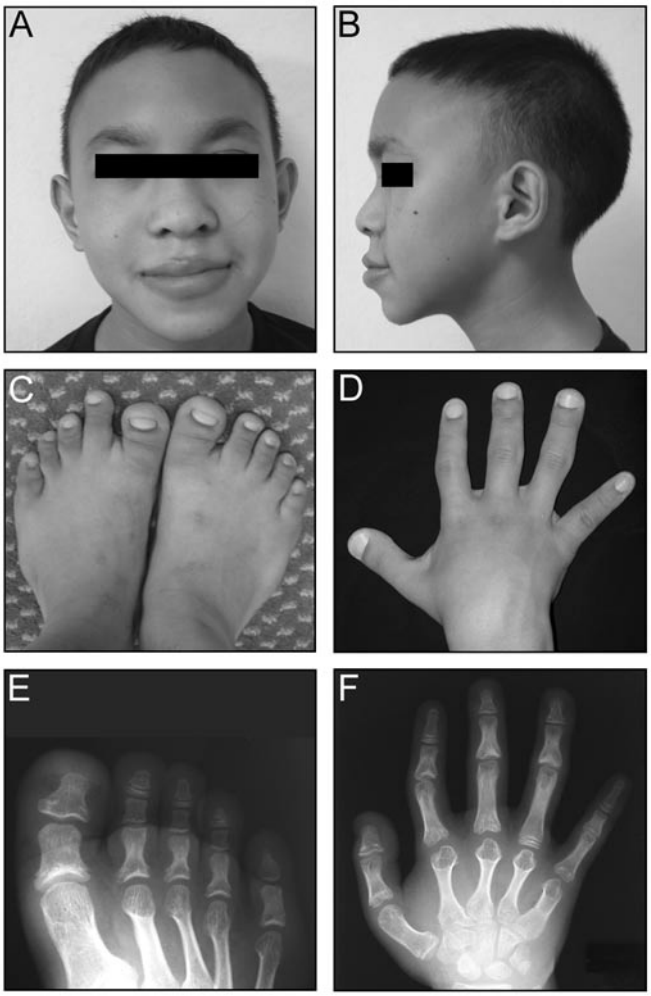


Figure 2. TRPS clinical features of a 17-year-old Thai boy. **(A)** Frontal picture of the participant showing a high hairline and broad alae of nose. **(B)** Sagittal view of the individual. **(C)** Feet with very broad halluces. **(D)** Hand showing deviation of fingers at proximal interphalangeal joints. **(E,F)** X-rays of, respectively, foot and hand showing multiple cone-shaped epiphyses.

Trps1$^{\Delta gt}$ mutants (E12.5 and E13.5). An abnormally arched palate was previously described for both heterozygous and homozygous *Trps1* mutants (Malik *et al.*, 2002). We observed a cleft palate in E18.5 homozygous mutants (Fig. 1I), which has not been reported previously.

Mutation in *TRPS1* Associated with Supernumerary Teeth

We identified dental abnormalities in a 17-year-old Thai male displaying clinical features of TRPS. He showed slow-growing hair, high-frontal hairline, and broad alae of the nose (Fig. 2A). In addition, clinobrachydactyly of hands and feet, with very broad thumbs, was noted (Figs. 2C, 2D). Radiographic examination of hands and feet revealed short metacarpals, multiple cone-shaped epiphyses of phalanges, and short middle phalanges of hands and feet (Figs. 2E, 2F). Height and intelligence were normal. He did not have the TRPS-typical thin lips. The participant was the only child of non-consanguineous parents, and his half sister showed no sign of TRPS.

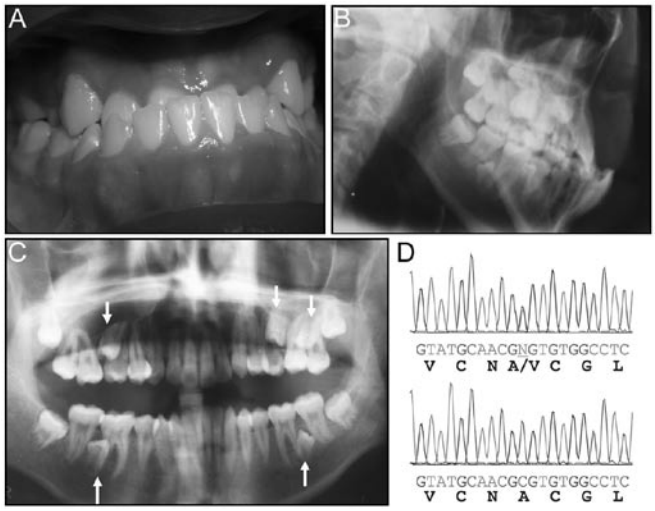


Figure 3. Mandibular prognathism and supernumerary teeth observed in a 17-year-old Thai male with TRPS. **(A)** Mandibular prognathism observed at 14 yrs old. **(B)** X-ray at age 17 showing a very prognathic mandible. **(C)** Panoramic x-ray at age 17 shows 5 non-erupted supernumerary teeth exhibiting premolar-like shape (arrows). **(D)** Electrophoretograms of the sequencing of PCR products. A heterozygous C>T mutation was found at nucleotide position 2756.

Oral examination showed severe mandibular prognathism (Fig. 3A) and rounded maxillary premolars and molars, while the mandibular teeth appeared normal. Cephalometric analysis showed a normally positioned maxilla, very prognathic mandible, high mandibular plane angle, increased gonial angle, reduced facial depth length, and long anterior facial height (Fig. 3B). Panoramic radiograph showed 5 supernumerary teeth in the premolar and molar areas of the maxilla and mandible. They all appeared to have the morphology of premolars (Fig. 3C).

Mutation screening of the participant was achieved by PCR amplification and direct sequencing of a DNA sample. A heterozygous 2756C>T mutation was found in exon 6 of *TRPS1* (Fig. 3D). This mutation causes the amino acid substitution alanine to valine at position 919 (A919V). This conservative amino acid change occurs in the zinc-coordinating domain of the GATA-type zinc-finger. This particular alanine is evolutionarily conserved in all mammalian GATA and GATA-like Zfn, suggesting its importance in normal GATA zinc structure and function (Hilton *et al.*, 2002).

DISCUSSION

In the mouse, expression of *Trps1* is found in the mesenchymal cells of the developing tooth germs, first in the dental mesenchyme condensing around the developing teeth, and later in the dental follicle and pre-odontoblasts. Interestingly, *Trps1* expression is down-regulated in mature dentin-secreting odontoblasts, suggesting a transient role of *Trps1* in odontoblast differentiation. Strong expression of *Trps1* in the oral mesenchyme at the time of tooth initiation (E11.5) is consistent with a possible role of *Trps1* in early tooth determination. In the mandible, this early expression of *Trps1* may also be significant regarding the mandibular prognathism observed in

our TRPS individual.

We have demonstrated that mice lacking the GATA domain of *Trps1* can display a cleft palate, a feature not reported previously. A high-arched palate has been described for several persons with TRPS I (Goodman *et al.*, 1981; Howell and Wynne-Davies, 1986), and one submucous cleft palate was reported in a person with TRPS II (Morioka *et al.*, 1999). Otherwise, *Trps1* mutant mice had a normal dental formula, and tooth development proceeded normally.

So far, all missense mutations found in the GATA DNA-binding domain of TRPS1 have been associated with the more severe TRPS type III (Lüdecke *et al.*, 2001; Kobayashi *et al.*, 2002). Two different missense mutations at position 919 are among them. The A919T mutation was described in four individuals from three families (Lüdecke *et al.*, 2001) diagnosed as TRPS III. The same mutation as in our patient (A919V) has also been described in a person with TRPS III (case T0202, Hilton *et al.*, 2002). Our patient has a normal stature, but short hands with short metacarpals, which is an intermediate phenotype between TRPS I and TRPS III. In addition, he displays several supernumerary teeth in the molar region and has mandibular prognathism. Unfortunately, the dental status of none of the persons with missense mutations at amino acid position 919 has been reported. And vice versa, none of the rare individuals reported in the literature as having supernumerary teeth (Goodman *et al.*, 1981; Peterson and Thomas, 2000; Karacay *et al.*, 2007) has been molecularly examined. Thus, the identification of the *TRPS1* mutation in the present case may help us understand the genetic basis of supernumerary teeth in persons with TRPS.

The IKAROS-like zinc-finger of TRPS1, located at the C-terminal end of the protein, is known to mediate protein-protein interactions with other IKAROS isoforms (Sun *et al.*, 1996). Homo- or heterodimerization may therefore be necessary for TRPS1 normal function. The A919V mutation presumably affects the DNA-binding affinity of the GATA zinc-finger, and TRPS1 with a missense mutation may compete with wild-type TRPS1 in a multimeric transcription control complex. Alternatively, the A919V missense mutation may cause a gain of function of TRPS1 and be responsible for the less common TRPS clinical manifestations, such as supernumerary teeth and mandibular prognathism.

It is therefore possible that mice lacking the GATA domain of TRPS1 are not a suitable model for addressing the function of TRPS1 during tooth development, since deletion of the GATA domain of mouse *Trps1* does not mimic the human missense mutation in the person with supernumerary teeth. The absence of a tooth phenotype in the *Trps1* mutant mice is overall consistent with the majority of people with TRPS with non-sense mutations who do not exhibit any obvious dental anomalies. The missense mutation we identified in the person with TRPS with additional dental anomalies is unusual and has been previously reported in only one other person, the dental status of whom was not described. The strong, localized expression of *Trps1* during tooth development is suggestive of a role that is clearly not revealed by this particular missense mutation. An amino acid change in an important DNA-binding domain could result in an increase or decrease in binding or alter binding specificity. The rather mild "facial" TRPS features of our participant, together with the unusual tooth phenotype, which suggest a hypomorphic loss of function together with a change in DNA-binding target specificity, might be the consequence of the A-V mutation.

It was recently shown that TRPS1 can bind the promoter of *RUNX2* and inhibit the activity of the *RUNX2* promoter *in vitro* (Napierala *et al.*, 2005). RUNX2 is a transcription factor that belongs to the runt domain family of proteins. Heterozygous mutations of *RUNX2* in humans lead to cleidocranial dysplasia (CCD; OMIM 119600), an autosomal-dominant skeletal dysplasia characterized by short stature, hypoplastic clavicles, large fontanelles, and orofacial abnormalities (Mundlos, 1999). The palate is often highly arched, and cleft palates have been described. Dental abnormalities include delayed eruption of permanent teeth associated with the presence of a very large number of unerupted supernumerary teeth, which appear to be morphologically normal. During mouse tooth development, *Runx2* transcripts are found in abundance in the dental mesenchyme at the bud and cap stages. At later stages, *Runx2* expression is absent in odontoblasts, but strong in the dental follicle (Yamashiro *et al.*, 2002). Co-expression of *Runx2* and *TRPS1* in dental mesenchymal cells is consistent with a possible interaction between *Runx2* and *TRPS1* during early tooth development. It is striking that extra teeth are observed both in persons with CCD and in those with unusual TRPS. If, during tooth development, *Trps1* belongs to the same pathway as *Runx2* and acts as a repressor of *Runx2*, it is likely that the TRPS1 mutation A919V results in a gain of function of TRPS1, since the supernumerary teeth observed in our TRPS participant mimic the dental phenotype of persons with RUNX2$^{+/-}$.

In summary, expression of *Trps1* during mouse tooth development and the discovery of a *TRPS1* missense mutation in a person with TRPS with supernumerary teeth suggest a new role for TRPS1 in tooth morphogenesis. It is crucial to identify the function of mutant A919V TRPS1 if we are to understand the basis of the generation of extra teeth. Further analyses of TRPS1 mutations in other individuals, combining clinical features of TRPS with dental anomalies, is likely to shed new light on TRPS1 function.

ACKNOWLEDGMENTS

We are grateful to the participant and his family for allowing us to use his medical and dental information for publication. The research is supported by The Thailand Research Fund (TRF) to P.K., Research Councils UK (RCUK) to I.M., and the Deutsche Forschungsgemeinschaft (DFG) to H.-J.L.

REFERENCES

Bühler EM, Buhler UK, Stalder GR, Jani L, Jurik LP (1980). Chromosome deletion and multiple cartilaginous exostoses. *Eur J Pediatr* 133:163-166.

Bühler EM, Buhler UK, Beutler C, Fessler R (1987). A final word on the tricho-rhino-phalangeal syndromes. *Clin Genet* 31:273-275.

Giedion A (1966). Das Tricho-rhino-phalangeal Syndrom. *Helv Paediatr Acta* 21:475-485.

Giedion A, Burdea M, Fruchter Z, Meloni T, Trosc V (1973). Autosomal dominant transmission of the tricho-rhino-phalangeal syndrome: report of 4 unrelated families, review of 60 cases. *Helv Paediatr Acta* 28:249-259.

Goodman RM, Trilling R, Hertz M, Horoszowski H, Merlob P, Reisner S (1981). New clinical observations in the trichorhinophalangeal syndrome. *J Craniofac Genet Dev Biol* 1:15-29.

Gorlin RJ, Cohen MM, Hennekam R (2001). Tricho-rhino-phalangeal syndromes. In: Syndromes of the head and neck. New York: Oxford University Press, pp. 1005-1009.

Hilton MJ, Sawyer JM, Gutierrez L, Hogart A, Kung TC, Wells DE (2002). Analysis of novel and recurrent mutations responsible for the tricho-rhino-phalangeal syndromes. *J Hum Genet* 47:103-106.

Howell CJ, Wynne-Davies R (1986). The tricho-rhino-phalangeal syndrome. A report of 14 cases in 7 kindreds. *J Bone Joint Surg B* 68:311-314.

Hussels IE (1971). Trichorhinophalangeal syndrome in two sibs. *Birth Defects Orig Artic Ser* 7:301-303.

Karacay S, Saygun I, Tunca Y, Imirzalioglu N, Guvenc G (2007). Clinical and intraoral findings of a patient with tricho-rhino-phalangeal syndrome type I. *J Indian Soc Pedod Prev Dent* 25:43-45.

Kobayashi H, Hino M, Shimodahira M, Iwakura T, Ishihara T, Ikekubo K, *et al.* (2002). Missense mutation of TRPS1 in a family of tricho-rhino-phalangeal syndrome type III. *Am J Med Genet* 107:26-29.

Kunath M, Lüdecke HJ, Vortkamp A (2002). Expression of *Trps1* during mouse embryonic development. *Mech Dev* 119(Suppl 1):117-120.

Langer LO Jr, Krassikoff N, Laxova R, Scheer-Williams M, Lutter LD, Gorlin RJ, *et al.* (1984). The tricho-rhino-phalangeal syndrome with exostoses (or Langer-Giedion syndrome): four additional patients without mental retardation and review of the literature. *Am J Med Genet* 19:81-112.

Lüdecke HJ, Wagner MJ, Nardmann J, La Pillo B, Parrish JE, Willems PJ, *et al.* (1995). Molecular dissection of a contiguous gene syndrome: localization of the genes involved in the Langer-Giedion syndrome. *Hum Mol Genet* 4:31-36.

Lüdecke HJ, Schmidt O, Nardmann J, von Holtum D, Meinecke P, Muente M, *et al.* (1999). Genes and chromosomal breakpoints in the Langer-Giedion syndrome region on human chromosome 8. *Hum Genet* 105:619-628.

Ludecke HJ, Schaper J, Meinecke P, Momeni P, Gross S, von Holtum D, *et al.* (2001). Genotypic and phenotypic spectrum in tricho-rhino-phalangeal syndrome types I and III. *Am J Hum Genet* 68:81-91.

Malik TH, Shoichet SA, Latham P, Kroll TG, Peters LL, Shivdasani RA (2001). Transcriptional repression and developmental functions of the atypical vertebrate GATA protein TRPS1. *EMBO J* 20:1715-1725.

Malik TH, von Stechow D, Bronson RT, Shivdasani RA (2002). Deletion of the GATA domain of TRPS1 causes an absence of facial hair and provides new insights into the bone disorder in inherited tricho-rhino-phalangeal syndromes. *Mol Cell Biol* 22:8592-8600.

Miletich I, Cobourne MT, Abdeen M, Sharpe PT (2005). Expression of the Hedgehog antagonists Rab23 and Slimb/βTrCP during mouse tooth development. *Arch Oral Biol* 50:147-151.

Momeni P, Glockner G, Schmidt O, von Holtum D, Albrecht B, Gillessen-Kaesbach G, *et al.* (2000). Mutations in a new gene, encoding a zinc-finger protein, cause tricho-rhino-phalangeal syndrome type I. *Nat Genet* 24:71-74.

Morioka D, Suse T, Shimizu Y, Ohkubo F, Hosaka Y (1999). Langer-Giedion syndrome associated with submucous cleft palate. *Plast Reconstr Surg* 103:1458-1463.

Mundlos S (1999). Cleidocranial dysplasia: clinical and molecular genetics. *J Med Genet* 36:177-182.

Napierala D, Garcia-Rojas X, Sam K, Wakui K, Chen C, Mendoza-Londono R, *et al.* (2005). Mutations and promoter SNPs in RUNX2, a transcriptional regulator of bone formation. *Mol Genet Metab* 86:257-268.

Niikawa N, Kamei T (1986). The Sugio-Kajii syndrome, proposed tricho-rhino-phalangeal syndrome type III. *Am J Med Genet* 24:759-760.

Pashayan HM, Solomon LM, Chan G (1974). The tricho-rhino-phalangeal syndrome. *Am J Dis Child* 127:257-261.

Peterson A, Thomas PS (2000). Abnormal modelling of the humeral head in the tricho-rhino-phalangeal syndrome: a new radiological observation. *Australas Radiol* 44:325-327.

Sun L, Liu A, Georgopoulos K (1996). Zinc finger-mediated protein interactions modulate *Ikaros* activity, a molecular control of lymphocyte development. *EMBO J* 15:5358-5369.

Wilkinson DG (1992). In situ hybridisation: a practical approach. Oxford, UK: Oxford University Press.

Yamamoto Y, Oguro N, Miyao M, Yanagisawa M (1989). Tricho-rhino-phalangeal syndrome type I with severe mental retardation due to interstitial deletion of 8q23.3-24.13. *Am J Med Genet* 32:133-135.

Yamashiro T, Aberg T, Levanon D, Groner Y, Thesleff I (2002). Expression of *Runx1, -2, -3* during tooth, palate and craniofacial bone development. *Mech Dev* 119(Suppl 1):107-110.

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

The Smallest Teeth in the World are Caused by Mutations in the *PCNT* Gene

Piranit Kantaputra,[1]* Pranoot Tanpaiboon,[2] Thantrira Porntaveetus,[3] Atsushi Ohazama,[3] Paul Sharpe,[3] Anita Rauch,[4] Atiwat Hussadaloy,[5] and Christian T. Thiel[6]

[1]Division of Pediatric Dentistry, Department of Orthodontics and Pediatric Dentistry, Craniofacial Genetics Laboratory, Faculty of Dentistry, Chiang Mai University; Dentaland Clinic, Chiang Mai, Thailand

[2]Division of Genetics and Metabolism, Children's National Medical Center, Washington, DC

[3]Department of Craniofacial Development and Comprehensive Biomedical Research Centre, Dental Institute, Kings College London, London, UK

[4]Institute of Medical Genetics, University of Zurich, Schwerzenbach-Zurich, Switzerland

[5]Nice Smiles Dental Clinic, Muang District, Khonkaen, Thailand

[6]Institute of Human Genetics, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany

Received 16 November 2010; Accepted 10 February 2011

We report a follow up study on two MOPD II Thai families with severe dental anomalies and hypoplastic alveolar bone. Striking dental anomalies comprise severe microdontia, opalescent and abnormally shaped teeth, and rootless molars. As a result of severe hypoplastic alveolar bone, most permanent teeth have been lost. Mutation analysis of *PCNT* revealed 2 novel mutations (p.Lys3154del and p.Glu1154X) and a recurrent mutation (p.Pro1923X). Teeth of the patient who carried a homozygous novel mutation of p.Glu1154X are probably the smallest ever reported. The sizes of the mandibular permanent incisors and all premolars were approximately 2-2.5 mm, mesiodistally. All previously reported, *PCNT* mutations have been described to cause premature truncation of the pericentrin protein. p.Lys3154del mutation was unique as it was pathogenic as a result of missing only a single amino acid. *In situ* hybridization of Pcnt shows its expression in the epithelium and mesenchyme during early stages of rodent tooth development. It is evident that PCNT has crucial role in tooth development. The permanent dentition is more severely affected than the one. This implies that PCNT appears to have more role in the development of the permanent dentition. As pericentrin is a critical centrosomal protein, the dental phenotype found in MOPD II patients is postulated to be the consequence of loss of microtubule integrity which leads to defective centrosome function.

Key words: microdontia; pericentrin; rootless teeth; hypoplastic alveolar bone; primordial dwarfism; opalescent teeth; malformed teeth; pigmentation anomalies; sparse hair; Seckel syndrome

How to Cite this Article:
Kantaputra P, Tanpaiboon P, Porntaveetus T, Ohazama A, Sharpe P, Rauch A, Hussadaloy A, Thiel CT. 2011. The smallest teeth in the world are caused by mutations in the *PCNT* gene.
Am J Med Genet Part A 9999A: 1–6.

INTRODUCTION

Microcephalic osteodysplastic primordial dwarfism type II (MOPD II; OMIM 210720) is a rare autosomal recessive inherited form of primordial dwarfism, characterized by severe pre- and postnatal growth retardation, relatively proportionate head size at birth, but pronounced microcephaly in adulthood. Bone dysplasia is progressive with metaphyseal and epiphyseal changes, and progressive joint hypermobility with occasional dislocation of the knees, radial heads, and hips. Affected patients present with distinct craniofacial features, consisting of prominent nose with hypoplastic alae nasi, small and dysplastic pinnae, small mouth, and sparse hair. Truncal obesity, farsightedness, esotropia, astigmatism, high-pitched nasal voice, cheerful personality, mild developmental delay, hypo- and

Grant sponsor: The Thailand Research Fund (to P.K.); Grant sponsor: European Community's Sixth Framework Programme (to P.K.); Grant number: LSHBCT-2005-019067; Grant sponsor: NIHR comprehensive Biomedical Research Centre at Guy's and St Thomas' (to P.T.S.); Grant sponsor: NHS foundation Trust and King's College London (to P.T.S.); Grant sponsor: Bundesministerium für Bildung und Forschung (BMBF) network (to A.R.); Grant number: SKELNET GFGM01141901.
*Correspondence to:
Piranit Kantaputra, Division of Pediatric Dentistry, Department of Orthodontics and Pediatric Dentistry, Craniofacial Genetics Laboratory, Faculty of Dentistry, Chiang Mai University; Dentaland Clinic, Chiang Mai 50200, Thailand. E-mail: dentaland17@gmail.com
Published online 00 Month 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/ajmg.a.33984

hyperpigmentation of skin and acanthosis nigricans around the neck are further hallmarks of MOPD II. A number of patients have been reported with life threatening CNS hemorrhages and strokes early in life, which are associated with aneurysms of the CNS arteries and/or Moya–Moya disease. These may lead to dilatation or aneurysm of the CNS arteries and Moya–Moya disease [Brancati et al., 2005; Bober et al., 2010]. Since abnormalities of CNS vessels are common in MOPD II patients, it has also been postulated that pericentrin may have important roles in the development of cerebral vessels [Piane et al., 2009]. Interfamilial and intrafamilial variability of the phenotype especially that of teeth have been described. Even though, the phenotype of MOPD II shares some similarities with Seckel syndrome, it is clearly distinguishable from Seckel syndrome by the severity of growth retardation, the presence of skeleton abnormalities, and the mild/absent mental retardation [Hall et al., 2004] and dental anomalies [Kantaputra et al., 2004]. So far, only loss of function mutations and deletions in the *PCNT* gene have been reported to be associated with MOPD II [Rauch et al., 2008; Willems et al., 2010]. In 2002 and 2004, Kantaputra et al. reported two MOPD Thai families with disproportionately and extremely small teeth. Some teeth have been reported to be even opalescent and rootless. As a result of having extreme and unique dental phenotype, these patients were originally stated as having a distinct syndrome (OMIM %607561: MOPD with tooth abnormalities) [Kantaputra, 2002; Kantaputra et al., 2004]. Here, we report a follow up study on both families with the emphasis on dental anomalies and the causative mutations in PCNT.

CLINICAL REPORT

These two MOPD families were originally and mistakenly reported as having a distinct MOPD syndrome [Kantaputra, 2002; Kantaputra et al., 2004].

Family 1

In 2002 Kantaputra reported MOPD II with severe dental anomalies in an 18-year-old Thai man and his 16-year-old sister, who are offspring of healthy, nonconsanguineous parents. The striking and quite distinctive dental anomalies consist of severe microdontia, opalescent teeth, abnormally shaped teeth, and rootless molars. While all teeth were small, the mandibular premolars were unusually small and malformed. Teeth were loose and the occlusal and incisal surfaces wore off very quickly [Kantaputra, 2002]. A follow-up study was performed 8 years after the original one, when they were 26 and 24 years old, respectively (Figs. 1A,B,F and 2A–F). Except for the MOPD II features, they had been healthy. The brother lost all of his permanent teeth and the alveolar bone was severely hypoplastic (Fig. 1C). The younger sister had five remaining permanent teeth with one unerupted right mandibular

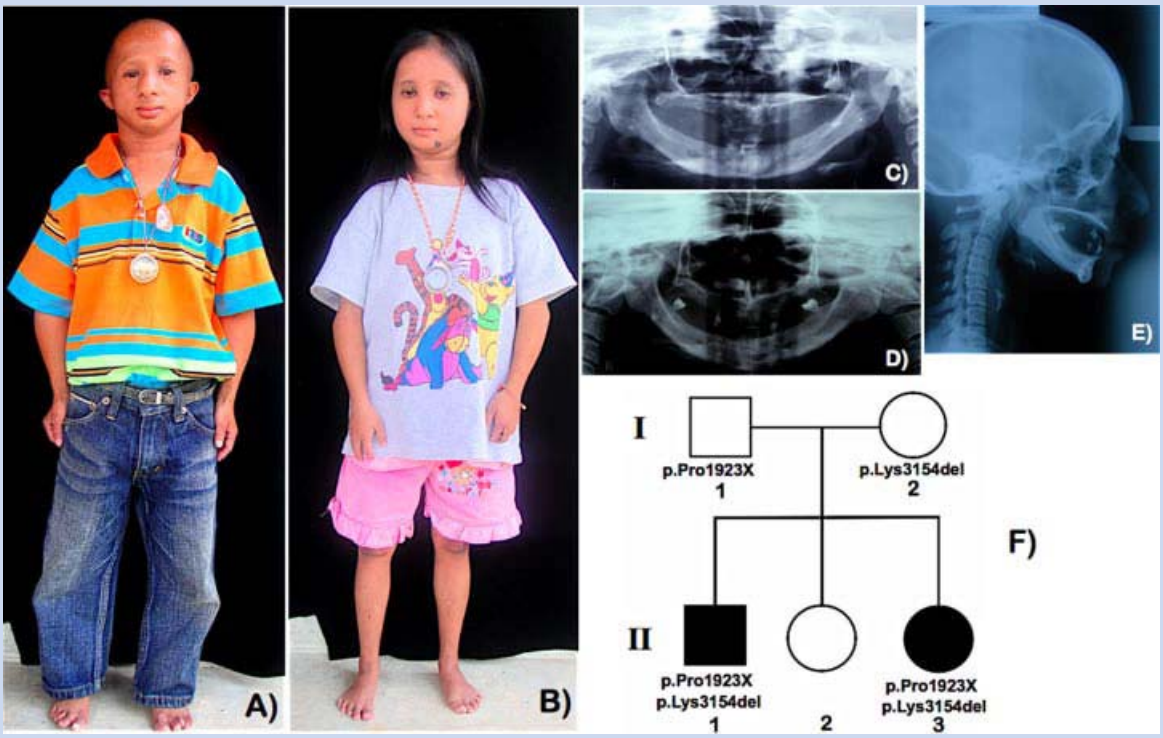


FIG. 1. Family 1. A: Affected man at age 26 years. B: Affected woman at age 24 years. C: Panoramic radiograph of the affected man at age 26 years shows absence of teeth and severely hypoplastic alveolar bone. D: Panoramic radiograph of the affected woman shows five remaining permanent teeth with unerupted premolar. E: Lateral cephalograph of the affected woman show severely hypoplastic alveolar bone. F: Pedigree of Family 1.

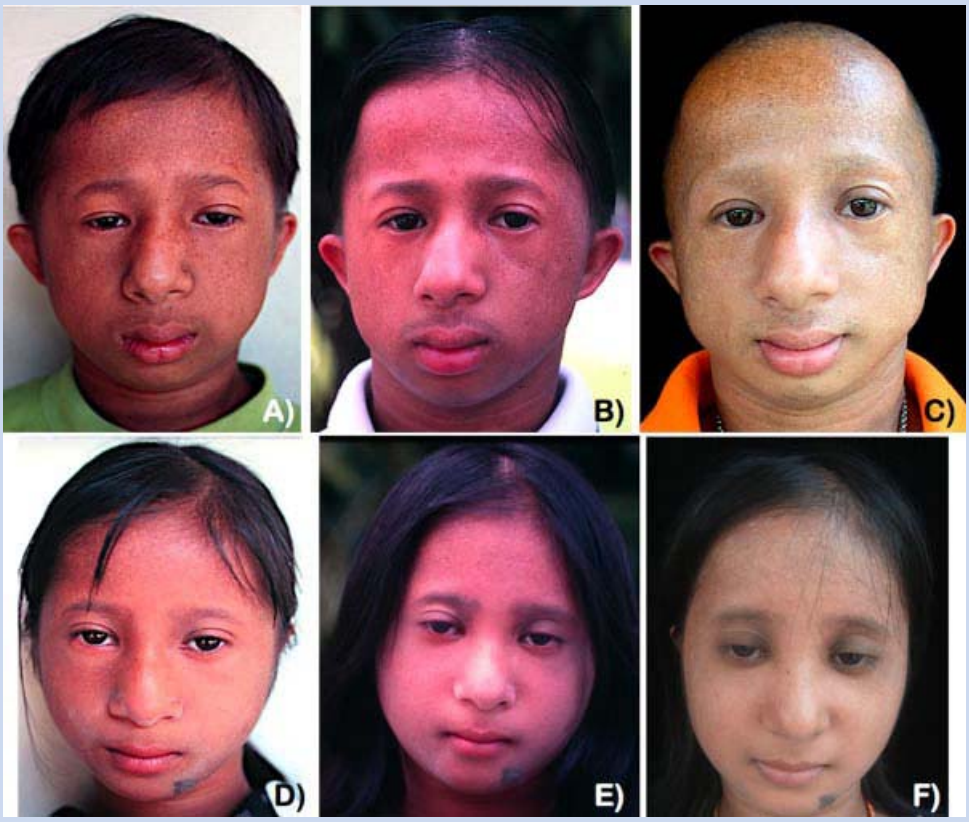


FIG. 2. Family 1. Affected man at ages (A) 14 years, (B)18 years, and (C) 26 years. He had his hair shaved. Affected woman at ages (D) 12, (E) 16, and (F) 24 years. Note hyper/hypopigmentation of skin.

premolar. The two remaining molars were rootless. Radiographically the alveolar bone was not observed and sella turcica appeared large (Fig. 1D,E).

Family 2

A 7-year-old Thai boy and his 3rd degree 9-year-old female cousin were reported in 2004 as having MOPD with severe microdontia and skin anomalies (Fig. 3A–E) [Kantaputra et al., 2004]. They were very cheerful children with friendly personality. A second clinical evaluation was performed when the boy and girl were 10 and 12 years old, respectively (Fig. 3A). As previously reported, his primary teeth were of normal sizes but the permanent teeth were extremely small. The sizes of the incisors and premolars were 2–2.5 mm mesiodistally [Kantaputra et al., 2004]. He had lost many permanent teeth painlessly and the remaining permanent teeth were significantly small. Interestingly all first permanent molars were of normal sizes and rootless (Fig. 3B–D). Unfortunately the boy died at the age of 11 years of unknown cause. The parents refused autopsy. The girl was otherwise healthy.

Regarding the skin, all of our MOPD II patients had hypo- and hyperpigmented skin, dry skin and the skin appeared darker as they aged. Multiple creases of the palms and soles were evident. Acanthosis nigricans was present only in the girl of Family 2.

METHODS

Mutation Analysis

The study was conducted with the consents of all family members and was approved by the ethics committee of the Faculty of Dentistry, Chiang Mai University. Blood was collected and DNA was extracted by routine techniques. Direct gene sequencing of all 47 coding exons and their flanking introns of *PCNT* were performed bidirectionally using methods previously described [Rauch et al., 2008]. Sequences were compared with the NCBI genomic sequence NM006031 with the SeqPilot software (JSI medical systems).

In Situ Hybridization

CD1 mouse heads were fixed in 4% paraformaldehyde, wax embedded and serially sectioned at 7 µm. Sections were split over 5–10 slides and prepared for radioactive in situ hybridization. Decalcification using 0.5 M ethylenediaminetetra acetic acid (pH 7.6) was performed after fixation of E18.5 mice. Radioactive section in situ hybridization using 35S-UTP radiolabelled riboprobes was carried out as described previously [Ohazama et al., 2008].

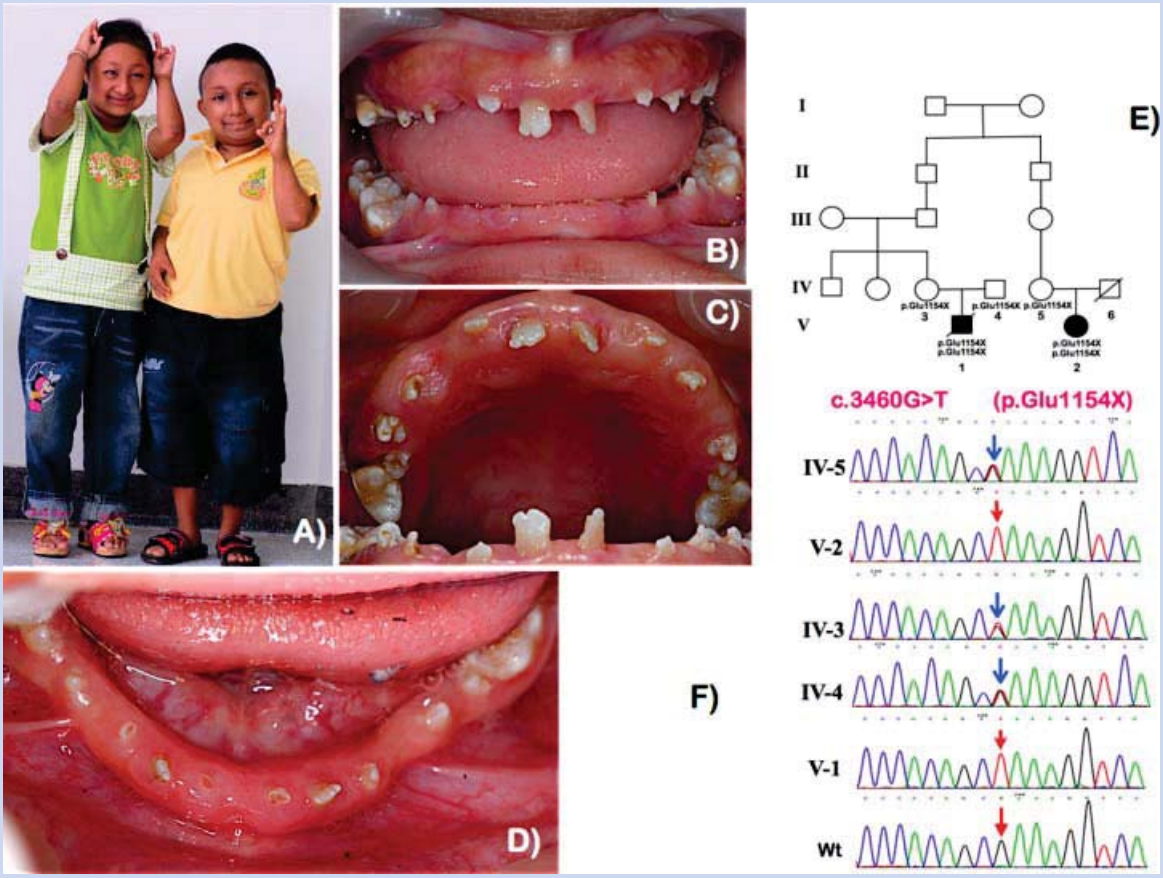


FIG. 3. Family 2. A: Affected girl and boy at ages 12 and 10, respectively. B–D: Remaining teeth of the boy at age 10 years. Note severe microdontia with abnormally shaped teeth. The first permanent molars are of normal sizes and shapes. E: Pedigree of the family. Each parent is heterozygous of p.E1154X. The affected boy and girl are homozygous of the mutation. F: Chromatograms show heterozygous mutation c.3460G>T in the parents and it is homozygous in the affected boy and girl.

RESULTS

Family 1

Direct sequencing of the coding regions and the flanking introns of *PCNT* gene revealed the heterozygous mutation c.9460_9462delAAG in exon 43 of the unaffected mother. The mutation is predicted to cause deletion of a single amino acid (p.Lys3154del). A heterozygous nonsense mutation c.5767C>T in exon 28, leading to premature truncation of pericentrin (p.Pro1923X) was detected in the unaffected father. The affected children were compound heteroizygous for both mutations.

Family 2

Mutation analysis of *PCNT* of the affected children revealed homozygous mutation c.3460G>T in the exon 17 in the affected boy and girl. Each parent was heterozygous of the mutation. This mutation is predicted to cause a premature stop codon (p.Glu1154X), leading to premature truncation of pericentrin (Fig. 3E,F). These three variants were not detected in 100 normal controls.

PCNT Expression During Tooth Development

We carried out comparative *in situ* hybridization analysis of lower molar tooth development at E10.5, E12.5, E13.5, E14.5, and E18.5 in wild-type mice. This period encompasses molar tooth development from initiation to the onset of cytodifferentiation. Initiation begins before the tooth anlagen is morphologically visible. The first signals are derived from tooth presumptive epithelium at E9.5. *Pcnt* expression could not be detected in presumptive tooth regions at E10.5 (data not shown). Thickening of the oral epithelium takes place from E12. *Pcnt* was expressed in the thickened tooth epithelium and mesenchyme at E12.5 (Fig. 4). By E13.5 the tooth epithelium invaginates into underlying mesenchyme to form the epithelial bud. *Pcnt* showed weak expression in the bud epithelium at E13.5 (Fig. 4). By E14.5 the bud basal epithelium develops into the internal and the external (outer) enamel epithelium, and the mesenchyme develops into the dental papillae and the dental follicle. *Pcnt* expression was observed in cap epithelium and dental papillae at E14.5 (Fig. 4). The terminal differentiation of dentin-forming odontoblasts from dental papilla cells and the enamel-forming ameloblasts from the internal epithelium occurs between

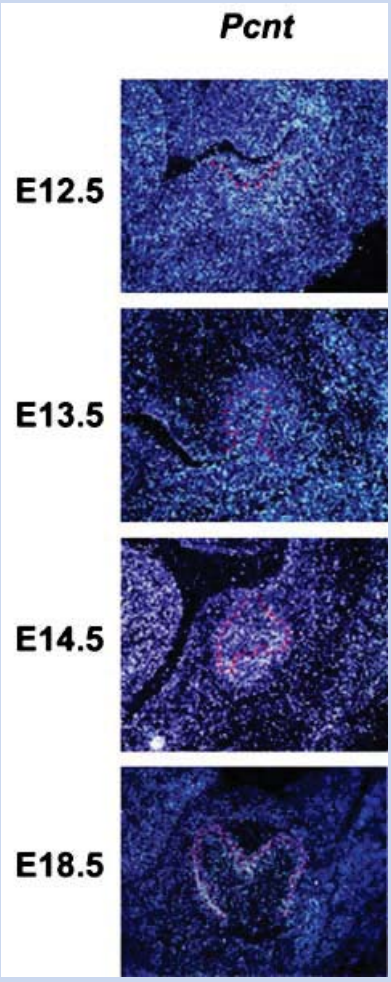


FIG. 4. ***Pcnt*** **expression in mouse tooth development. Radioactive** ***in situ*** **hybridization of** ***Pcnt*** **expression on frontal head sections at the positions of lower first molars. Tooth germ epithelium (E12.5–E14.5) and ameloblasts (E18.5) are outlined in red.**

E18 to P0. *Pcnt* was weakly expressed in both ameloblasts and odontoblasts at E18.5 (Fig. 4).

DISCUSSION

We report on a follow up study on two Thai families affected with MOPD II. We originally reported them as having a distinct syndrome as a result of their unique dental anomalies. The severity of microdontia and the hypoplasia of alveolar bone found in these Thai families are remarkable. However, we agree with Hall et al. [2004] that the phenotype found in these families just represented the variability of the phenotype of MOPD II [Hall et al., 2004]. Mutation analysis of PCNT in these families has revealed 2 novel and a recurrent mutations. The patients in Family 1 were affected with compound heterozygous mutations, p.Pro1923X and p.Lys3154del. Their unaffected father and mother were the carriers of p.Pro1923X and p.Lys3154del, respectively. p.Pro1923X has been reported in an Omani and Parkistani families [Rauch et al., 2008]. Here, the affected individuals also have small, widely spaced teeth and poorly formed enamel. All previously reported mutations in *PCNT* have been described to cause premature protein truncation and mRNA of those mutations were shown to be expressed, in some cases at near normal levels [Rauch et al., 2008; Piane et al., 2009; Willems et al., 2010]. The p.Lys3154del mutation found in Family 1 is novel and appears to be unique as it was pathogenic as a result of missing only a single amino acid. The lysine at position 3154 is a highly conserved amino acid in the PACT domain which has been shown to be important for the centrosomal binding of PCNT [Flory et al., 2000; Gillingham and Munro, 2000]. Therefore, the mutation most likely interferes with this very important function. The prediction of the effect of the deletion on protein function confirms a probable damaging effect (PolyPhen-2 score 0.996). The patients in Family 2 were affected with a homozygous novel mutation p.Glu1154X. All affected individuals had severe microdontia and severely hypoplastic alveolar bone. We are convinced that the permanent teeth found in the affected male (V-1) appeared to be the smallest permanent teeth ever been reported in the literature [Kantaputra et al., 2004]. The permanent teeth especially the premolars found in both affected patients of Family 2 were severely malformed (Fig. 3B–D). The p.Glu1154X mutation did not seem to have apparent effects on the development of primary teeth as they appeared to have normal sizes and eruption timing.

The *PCNT* gene encodes a critical centrosomal protein pericentrin (kendrin or pericentrin-B). It is mapped to 21q22.3, comprises 47 coding exons, and spans 122 kb of genomic sequences. Pericentrin is a large coiled-coil-protein which comprises 3,336 amino acid residues [Flory et al., 2000]. Pericentrin serves as a multifunctional scaffold for anchoring a wide range of controsomal proteins and protein complexes during cell division [Delaval and Doxsey, 2010] and interacts with γ-tubulin which is required for microtubule nucleation [Dictenberg et al., 1998].

The phenotype caused by *PCNT* mutations might reflect a role for spindle dysfunction in primordial dwarfism. The disruption of pericentrin would lead to mislocalization of proteins, which is crucial for microtubule nucleation or organization, and subsequently cause mitotic spindle defects, chromosome missegregation, mitotic failure, cell arrest, and/or cell death [Delaval and Doxsey, 2010]. The small sizes and the malformation of teeth found in patients with MOPD II might have been the consequences of loss of microtubule integrity which lead to defective centrosome function [Delaval and Doxsey, 2010]. Taking into account the dental phenotype of our patients and those of previously reported ones, we hypothesize that pericentrin has more important role in the development of the permanent dentition than that of the primary one. This has demonstrated that "All teeth are not created equal" and we could not use the mouse model to explain this interesting phenomenon because unlike human, mouse has only one dentition.

ACKNOWLEDGMENTS

We thank all family members for their kind cooperation and for allowing us to use their medical and dental information for the benefit of others. This work was supported by grants from The

Thailand Research Fund (TRF) and The European Community's Sixth Framework Programme (LSHBCT-2005-019067) to P.K.; the NIHR comprehensive Biomedical Research Centre at Guy's and St Thomas' NHS foundation Trust and King's College London to P.T.S.; Bundesministerium für Bildung und Forschung (BMBF) network grant SKELNET GFGM01141901 to A.R.; and the DFG grant TH896/4-1 to C.T.T.

REFERENCES

Brancati F, Castori M, Mingarelli R, Dallapiccola B. 2005. Majewski osteodysplastic primordial dwarfism type II (MOPD II) complicated by stroke: clinical report and review of cerebral vascular anomalies. Am J Med Genet Part A 139A:212–215.

Bober MB, Khan N, Kaplan J, Lewis K, Feinstein JA, Scott CI Jr., Steinberg GK. 2010. Majewski osteodysplastic primordial dwarfism type II (MOPD II): expanding the vascular phenotype. Am J Med Genet Part A 152A:960–965.

Delaval B, Doxsey SJ. 2010. Pericentrin in cellular function and disease. J Cell Biol 188:181–190.

Dictenberg JB, Zimmerman W, Sparks CA, Young A, Vidair C, Zheng Y, Carrington W, Fay FS, Doxsey SJ. 1998. Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. J Cell Biol 141:163–174.

Flory MR, Moser MJ, Monnat RJ Jr., Davis TN. 2000. Identification of a human centrosomal calmodulin-binding protein that shares homology with pericentrin. Proc Natl Acad Sci 97:5919–5923.

Gillingham AK, Munro S. 2000. The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. EMBO Rep 1:524–529.

Hall JG, Flora C, Scott CI Jr., Pauli RM, Tanaka KI. 2004. Majewski osteodysplastic primordial dwarfism type II (MOPD II): natural history and clinical findings. Am J Med Genet Part A 130A:55–72.

Kantaputra PN. 2002. Apparently new osteodysplastic and primordial short stature with severe microdontia, opalescent teeth, and rootless molars in two siblings. Am J Med Genet Part A 111:420–428.

Kantaputra PN, Tanpaiboon P, Unachak K, Praphanphoj V. 2004. Microcephalic osteodysplastic primordial dwarfism with severe microdontia and skin anomalies: confirmation of a new syndrome. Am J Med Genet Part A 130A:181–190.

Ohazama A, Johnson EB, Ota MS, Choi HY, Porntaveetus T, Oommen S, Itoh N, Eto K, Gritli-Linde A, Herz J, Sharpe PT. 2008. Lrp4 modulates extracellular integration of cell signaling pathways in development. PLoS One 3:e4092.

Piane M, Della Monica M, Piatelli G, Lulli P, Lonardo F, Chessa L, Scarano G. 2009. Majewski osteodysplastic primordial dwarfism type II (MOPD II) syndrome previously diagnosed as Seckel syndrome: report of a novel mutation of the *PCNT* gene. Am J Med Genet Part A 149A:2452–2456.

Rauch A, Thiel CT, Schindler D, Wick U, Crow YJ, Ekici AB, van Essen AJ, Goecke TO, Al-Gazali L, Chrzanowska KH, Zweier C, Brunner HG, Becker K, Curry CJ, Dallapiccola B, Devriendt K, DO?rfler A, Kinning E, Megarbane A, Meinecke P, Semple RK, Spranger S, Toutain A, Trembath RC, Voss E, Wilson L, Hennekam R, de Zegher F, Doerr HG, Reis A. 2008. Mutations in the pericentrin (*PCNT*) gene cause primordial dwarfism. Science 319:816–819.

Willems M, Geneviève D, Borck G, Baumann C, Baujat G, Bieth E, Edery P, Farra C, Gerard M, Héron D, Leheup B, Le Merrer M, Lyonnet S, Martin-Coignard D, Mathieu M, Thauvin-Robinet C, Verloes A, Colleaux L, Munnich A, Cormier-Daire V. 2010. Molecular analysis of pericentrin gene (*PCNT*) in a series of 24 Seckel/microcephalic osteodysplastic primordial dwarfism type II (MOPD II) families. J Med Genet 47:797–802.

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

c. 595-596 insC of FOXC2 Underlies Lymphedema, Distichiasis, Ptosis, Ankyloglossia, and Robin Sequence in a Thai Patient

Pranoot Tanpaiboon,[1]* Piranit Kantaputra,[2] Karn Wejathikul,[1] and Wirawit Piyamongkol[3]

[1]Faculty of Medicine, Department of Pediatrics, Chiang Mai University, Chiang Mai, Thailand

[2]Faculty of Dentistry, Craniofacial Genetics Laboratory, Department of Pediatric Dentistry, Chiang Mai, Thailand

[3]Faculty of Medicine, Department of Obstetrics and Gynaecology, Chiang Mai University, Chiang Mai, Thailand

Received 14 July 2009; Accepted 29 November 2009

Lymphedema–distichiasis syndrome is a rare primary lymphedema inherited as an autosomal dominant disorder. The characteristic features consist of late onset-lymphedema and distichiasis together with other occasionally seen features including varicose vein, cleft palate, ptosis, and congenital heart diseases. *FOXC2* is the gene found to be associated with this syndrome. We report here the first Thai patient who has characteristic features of this syndrome and the infrequently described features including ankyloglossia, and Robin sequence which consists of glossoptosis, cleft palate, and micrognathia. Mutation analysis of *FOXC2* revealed c. 595-596 insC.

Key words: ankyloglossia; distichiasis; *FOXC2*; glossoptosis; lymphedema; robin sequence

How to Cite this Article:
Tanpaiboon P, Kantaputra P, Wejathikul K, Piyamongkol W. 2010. c. 595-596 insC of FOXC2 Underlies lymphedema, distichiasis, ptosis, ankyloglossia, and robin sequence in a Thai patient.
Am J Med Genet Part A 152A:737–740.

INTRODUCTION

Lymphedema is a soft tissue swelling of the part of the body owing to the impairment of the lymphatic system to transport excess capillary filtrate containing liquid, macromolecules, and mobile cells back to the blood resulting in the excessive accumulation of protein-rich fluid in the interstitium [Erickson et al., 2001; Damstra and Mortimer, 2008]. The impairment or dysfunction of lymphatic system leads to an imbalance between lymph formation and its absorption into initial lymphatics. Lymphedema occurs predominantly in the lower limbs but it can also occur in other parts of the body [Bell et al., 2001].

Primary lymphedema occur rarely on idiopathic or developmental abnormalities of the lymphatic system [Ferrell et al., 1998]. It is rare in children, affecting 1.15/100,000 persons younger than 20 years [Bell et al., 2001]. This condition is classified into two subgroups, idiopathic and hereditary (familial) [Bell et al., 2001]. Hereditary lymphedema is of genetic origin and has been known to be associated with mutations of several genes. The phenotypes vary from isolated condition such as Milroy disease (OMIM #153100) or as a component of syndromes such as lymphedema–distichiasis (LD) syndrome, lymphedema–ptosis syndrome (OMIM #153000), yellow nail syndrome (OMIM #153300) or Turner syndrome.

Lymphedema–distichiasis syndrome (OMIM #153400) is a highly penetrant autosomal dominant disorder of a rare pubertal onset primary lymphedema. It is usually associated with distichiasis, which is the development of aberrant accessory eyelashes arising from the meibomian gland orifices along the posterior border of the lid margins [Traboulsi et al., 2002]. Associated findings of LD consist of ptosis (31%), varicose vein (25%), congenital heart diseases (6.8–10%), and cleft palate (4–10%). Scoliosis, renal anomalies, hydrocele, strabismus have infrequently been reported [Bell et al., 2001]. Mutations in *FOXC2* (formally known as MFH-1) gene, a member of forkhead/winged-helix family of transcription factor, have been reported to be responsible for the syndrome in a number of patients [Bell et al., 2001; Brice et al., 2002; Dellinger et al., 2008]. Familial cases have frequently been reported [Bell et al., 2001]. Here, we report on a Thai patient with a mutation in *FOXC2* and is affected with LD syndrome with ankyloglossia and Robin sequence including cleft palate, micrognathia, as well as glossoptosis.

*Correspondence to:
Dr. Pranoot Tanpaiboon, Division of Genetics and Metabolism, Children's National Medical Center, Washington, DC.
E-mail: tanpaiboon1@yahoo.com
Published online 22 February 2010 in Wiley InterScience (www.interscience.wiley.com)
DOI 10.1002/ajmg.a.33273

CLINICAL REPORT

A 12-year-old Thai boy presented with swelling of the left leg for 4 months. He is the only child of the nonconsanguineous family. He was born at term, delivered normally, and had normal body size for gestational age. Physical examination at age 12 years revealed bilateral mild ptosis, small mouth, crowding of teeth, ankyloglossia, glossoptosis, micrognathia, and cleft palate (Fig. 1). Hydrocoele of the right scrotal sac was detected and hydrocelectomy was performed. Two months after surgery, he developed swelling of the left leg with an episode of cellulitis which was later treated by intravenous antibiotic. After infection resolved, the left leg swelling still remained. The swollen left leg and foot were nonpitting and were covered with normal skin. Stimmer's sign (inability to pick up a fold of skin at the base of the second toe because of thickening or fibrosis of the tissues) was positive. No signs of chronic venous insufficiency were present. There was a double row of eyelashes (distichiasis) at the lower eyelids of both eyes with no signs of corneal irritation. Yellow nails, significant lymphadenopathy, or cardiac abnormalities were not detected. Physical examinations of both parents were unremarkable.

Result of laboratory studies including CBC, urinalysis, creatinine, and liver function test were normal. The thick blood film for filaria did not find the parasite. Venous duplex ultrasound showed no evidence of deep vein thrombosis. Abdominal computed tomography was unremarkable. Lymphoscintigraphic scan demonstrated increased lymph conducting pathways and dermal back flow indicating lymph reflux of the left leg (Fig. 2).

Informed consent was obtained from the father of the patient. DNA was extracted from venous blood according to standard procedures. The methods used to perform mutation analysis of *FOXC2* have previously been described [Bell et al., 2001]. Mutation analysis of *FOXC2* revealed a de novo heterozygous insertion of cytosine between nucleotide 595 and 596 (c. 595-596 insC) causing frameshift mutation and producing 263 novel amino acids. The mutation was not found in the father and the mother.

DISCUSSION

We report on a 12-year-old Thai boy with LD syndrome. His clinical findings consist of lymphedema, distichiasis, mild ptosis, cleft palate, and hydrocele. He also has micrognathia, glossoptosis, and ankyloglossia. Mutation analysis of FOXC2 demonstrated c. 595-596 insC. This mutation is predicted to cause a frameshift mutation and create 263 novel amino acids before truncating the protein in the carboxy-terminal region downstream to the forkhead domain (FHD). This mutation is expected to interfere with DNA binding or to disrupt C-terminal alpha-helices critical for transcription activation [Finegold et al., 2001].

FOXC2, a gene located on chromosome 16q24.3, is the only gene known to be responsible for LD [Bell et al., 2001; Brice et al., 2002]. It has single exon which is highly (70%) GC rich with no intron and produces a 2.2 kb transcript. The gene encodes FHD transcription factor genes, characterized by a conserved 100 amino acid DNA-binding motif that is found in various organisms ranging from yeasts to humans [Miura et al., 1997]. This gene has crucial roles in the development of the lymphatic primodial, capillary, lymphatic valve and network, developing eyelids and podocytes [Bell et al., 2001; Petrova et al., 2004].

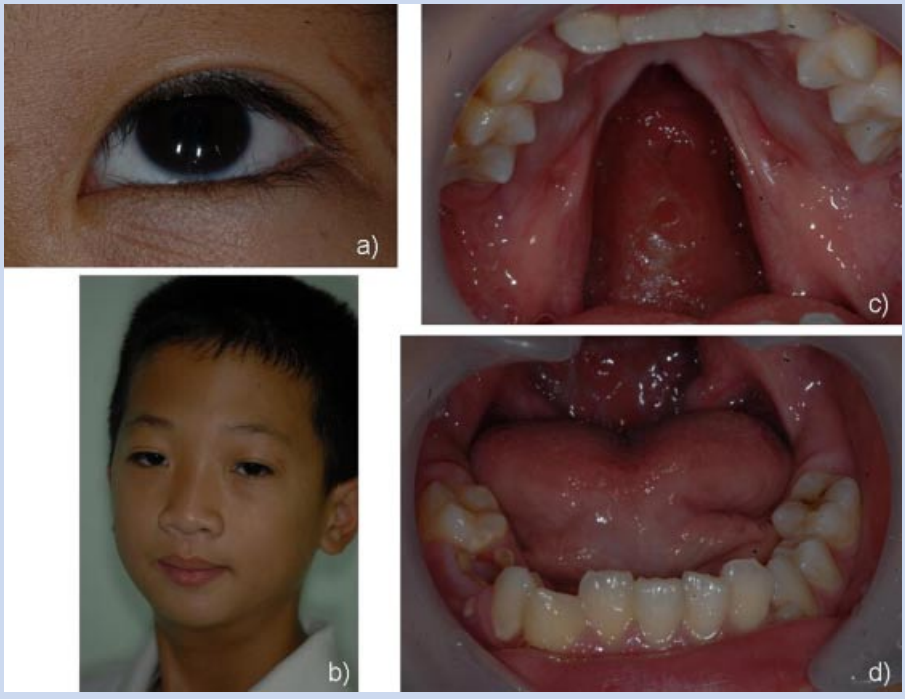


FIG. 1. a,b: Distichiasis. LD patient. c: Cleft palate. d: Glossoptosis, ankyloglossia, and cleft palate.

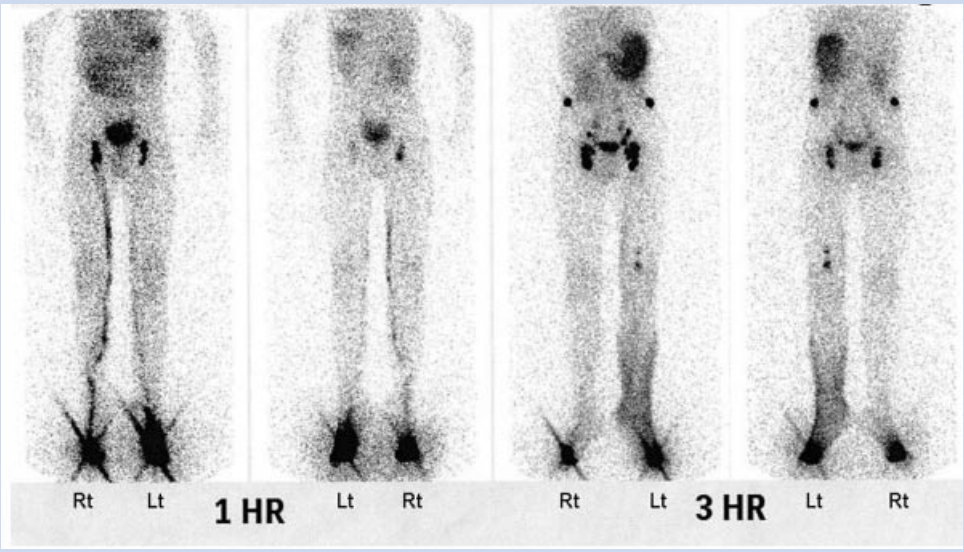


FIG. 2. Lymphoscintigraphic scan. A 1-hr image shows normal transportation of the tracer via the lymphatic vessels of both legs, thighs, groins, and pelvic. A delayed 3-hr image shows progressive filling of lymphatic vessels of superficial skin (dermal back flow) at the left leg (Lt).

Mutation sites have been discovered throughout the gene [Bell et al., 2001]. Interestingly, insertions and deletions creating truncated FOXC2 proteins were the predominant mutations (75% of reported mutations). Whereas, nonsense and missense mutations were less common [Bell et al., 2001; Brice et al., 2002; Dellinger et al., 2008].

Genotype/phenotype correlation has not been clearly demonstrated, therefore, it may conclude that all mutations including missense, insertion, and deletion mutations that produce LD effect protein function as truncating mutations resulting in haploinsufficiency [Bell et al., 2001; Erickson et al., 2001; Finegold et al., 2001; Ng et al., 2005]. This is supported by the lack of functional activity exhibited by *FOXC2* missense mutations [Berry et al., 2005].

Similar to the other transcription factor gene mutations, *FOXC2* mutations can cause many developing organ system malformations by directly effecting organ formation and/or indirectly influencing the other regulatory gene system involving organ formation. Although many animal studies demonstrated that Foxc2 haploinsufficiency leads to some of human LD phenotypes, there are some unanswered questions requiring more studies such as how Meibomian glands become hair follicles, why the onset of lymphedema usually occurred around 12-year old or why some patients developed DM.

Phenotypically, cleft palate is not a common finding in LD patients (4%) [Brice et al., 2002]. Moreover, ankyloglossia and Robin sequence consisting of cleft palate, micrognathia, and glossoptosis also are rarely reported as features of LD [O'Donnell and Collin, 1993; Temple and Collin, 1994].

In the conclusion, our patient was born with cleft palate and mild ptosis. Primary bilateral lymphedema first occurred at 12-year old. Asymptomatic distichiasis of both eyes was detected during physical examination. In addition to the characteristic features of the syndrome, he also has cleft palate, glossoptosis, and micrognathia indicating Robin sequence. This appears to be the first report to focus on Robin sequence. According to two of the characteristic features of LD, lymphedema which usually presents in puberty and distichiasis which may be asymptomatic, detecting Robin sequence in the early life may be an indicator to perform additional eye examination and may cause early disease recognition. Initiate the treatment early may prevent the complications from distichiasis and from irreversible fibrosclerotic changes in tissue surrounding lymphatic impairment area.

Both parents have no features of LD. Patient had nonsense mutation in FOXC2 which has been reported in Caucasian family but no mutation was found in the parents representing de novo mutation.

REFERENCES

Bell R, Brice G, Child AH, Murday VA, Mansour S, Sandy CJ, Collin JR, Brady AF, Callen DF, Burnand K, Mortimer P, Jeffery S. 2001. Analysis of lymphoedema-distichiasis families for *FOXC2* mutations reveals small insertions and deletions throughout the gene. Hum Genet 108:546–551.

Berry FB, Tamimi Y, Carle MV, Lehmann OJ, Walter MA. 2005. The establishment of a predictive mutational model of the forkhead domain through the analyses of FOXC2 missense mutations identified in patients with hereditary lymphedema with distichiasis. Hum Mol Genet 14: 2619–2627.

Brice G, Mansour S, Bell R, Collin JR, Child AH, Brady AF, Sarfarazi M, Burnand KG, Jeffery S, Mortimer P, Mortimer P, Murday VA. 2002. Analysis of the phenotypic abnormalities in lymphoedema-distichiasis syndrome in 74 patients with *FOXC2* mutations or linkage to 16q24. J Med Genet 39:478–483.

Damstra RJ, Mortimer PS. 2008. Diagnosis and therapy in children with lymphoedema. Phlebology 23:276–286.

Dellinger MT, Thome K, Bernas MJ, Erickson RP, Witte MH. 2008. Novel FOXC2 missense mutation identified in patient with lymphedema–distichiasis syndrome and review. Lymphology 41: 98–102.

Erickson RP, Dagenais SL, Caulder MS, Downs CA, Herman G, Jones MC, Kerstjens-Frederikse WS, Lidral AC, McDonald M, Nelson CC, Witte M, Glover TW. 2001. Clinical heterogeneity in lymphoedema-distichiasis with FOXC2 truncating mutations. J Med Genet 38:761–766.

Ferrell RE, Levinson KL, Esman JH, Kimak MA, Lawrence EC, Barmada MM, Finegold DN. 1998. Hereditary lymphedema: Evidence for linkage and genetic heterogeneity. Hum Mol Genet 7:2073–2078.

Finegold DN, Kimak MA, Lawrence EC, Levinson KL, Cherniske EM, Pober BR, Dunlap JW, Ferrell RE. 2001. Truncating mutations in FOXC2 cause multiple lymphedema syndromes. Hum Mol Genet 10:1185–1189.

Ng MY, Andrew T, Spector TD, Jeffery S. 2005. Linkage to the FOXC2 region of chromosome 16 for varicose veins in otherwise healthy, unselected sibling pairs. J Med Genet 42:235–239.

O'Donnell PA, Collin JR. 1993. Distichiasis: Management with cryotherapy to the posterior lamella. Br J Ophthalmol 77:289–292.

Petrova TV, Karpanen T, Norrmen C, Mellor R, Tamakoshi T, Finegold D, Ferrell R, Kerjaschki D, Mortimer P, Yla-Herttuala S, Miura N, Alitalo K. 2004. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. Nat Med 10:974–981.

Temple IK, Collin JR. 1994. Distichiasis-lymphoedema: A family report. Clin Dysmorphol 3:132–142.

Traboulsi EI, Al-Khayer K, Matsumoto M, Kimak MA, Crowe S, Wilson SE, Finegold DN, Ferrell RE, Meisler DM. 2002. Lymphedema–distichiasis syndrome and FOXC2 gene mutation. Am J Ophthalmol 134:592–596.