厚生科学研究費補助金（感覚器障害研究事業）
分担研究報告書

幹細胞から眼組織への分化誘導能に関する研究

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研究要旨：再生医療の材料として期待されている胚性幹(ES)細胞を効率良く眼組織へと分化誘導する実験系を開発する目的で、本年度は、眼形成のマスター遺伝子 PAX6 を用いたマウス ES 細胞から神経細胞への分化誘導系の開発を行った。PAX6 アイソフォーム 5a 型(PAX6-5a)を発現する ES 細胞株が強い神経細胞分化誘導能を示すことを見い出し、本神経細胞分化誘導系に関わる遺伝子をマイクロアレイ法により同定した。これらの結果は、再生医療に求められる幹細胞の試験管内培養法や眼組織への分化誘導系を確立する上で重要な知見を提供すると考えられる。

A. 研究目的

眼組織構築に期待されている ES 細胞の特性を明らかにし、将来の再生医療の基盤となる眼組織分化誘導系の開発を行う。

B. 研究方法

テトラサイクリンを用いた PAX6-5a 誘導可能なマウス ES 細胞を樹立して、神経細胞分化誘導能と神経細胞分化誘導に関与する遺伝子をマイクロアレイ法により検討した。細胞の取り扱いは、大学動物実験委員会の承認を得、そのプロトコールに従って遂行されている。

C. 研究成果

PAX6-5a が強い神経細胞分化誘導能を示すことを見い出した。また、本誘導系において、転写制御因子の bHLHb2 や POU5F1 が必須の役割を果たしていることが示唆された。

D. 考察

ES 細胞から神経細胞を調製する試みが世界中で試みられているが、眼形成マスター遺伝子 PAX6-5a も有望な手段であることが示された。また、PAX6-5a の生理的な役割は未だ不明の点が多いが、本 PAX6-5a 発現誘導 ES 細胞株は標的遺伝子の同定などその機能の解明に有用であると考えられる。

E. 結論

これまで我々は、PAX6-5a がニワトリの網膜形成誘導を有することを示してきたが、哺乳動物神経細胞への分化誘導能も有することを明らかにした。ES 細胞を用いた将来の網膜再生医療への重要なツールになると考えられる。

F. 健康危険情報

該当する危険は無し

G. 研究発表

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H. 知的財産権の出願・登録状況
1. 特許取得　　無し
2. 実用新案登録 無し
3. その他　　無し

厚生科学研究費補助金（感覚器障害研究事業）
分担研究報告書

眼の形態形成遺伝子を用いた網膜再生の研究

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研究要旨：鶏胚の網膜色素上皮細胞に眼の形態形成遺伝子 Pax6 を導入し、ほぼ完全な層構造をもつ神経網膜を作ることに成功した。作られた神経網膜の神経線維は、視神経を通って中枢へ投射していた。孵化直前のほぼ成熟した色素上皮細胞からも再生が可能であり、成熟組織にも幹細胞が存在することが示唆された。マウス胚でも同様に色素上皮細胞から神経網膜を再生することができたが、その層構造は不完全であった。これらの再生網膜は、変性した網膜の下に移植して視覚を復元する治療に応用することが期待される。

A．研究目的

眼の発生では多くの形態形成遺伝子が発見されたが、中でもPax6はすべての動物に存在し、ショウジョウバエ初期胚にほぼ完全な異所複眼を作成する能力があるので、眼の器官形成を全体的に支配するmaster control遺伝子であることが明らかになった。この異所眼作成は、脊椎動物ではアフリカツメガエル胚でも可能だが、角膜、水晶体、網膜などの眼組織を含むものの眼球形態は不完全である。しかし、再生医学においては眼球全体を作ることはできないにしても、一部の組織を作れる可能性がある。本研究では、網膜色素上皮細胞にPax6遺伝子を導入して神経網膜を再生させる研究を行った。

B．実験方法

1）鶏胚網膜色素上皮へのPax6遺伝子導入

鶏受精卵を孵卵器で発生を進め、2-20日胚の網膜色素上皮にPax6遺伝子を導入した。強制発現プラスミドベクターpCAGGSにPax6 cDNAを組み込み、これを電気穿孔法で鶏胚に導入した。Pax6遺伝子の変異体F258S、R26G、R128Cおよび機能抑制体En(s)-Pax6delC+の導入も行った。導入後、孵卵器で発生を進め、実体顕微鏡下と病理組織的に変化を検討した。

2）マウス胚網膜色素上皮へのPax6遺伝子導入

E12-15マウス胚の眼球を摘出し、網膜色素上皮近傍にPax6 cDNAを電気穿孔法で導入した。導入後、器官培養を行って発生を進め、実体顕微鏡下と病理組織的に変化を検討した。

C. 研究結果

鶏胚で Pax6 を導入した網膜色素上皮は、実体顕微鏡下で白色となって盛上り、組織は神経網膜に変化していた。多くはほぼ完全な層構造の網膜になっていたが、層の方向性はいずれも本来ある神経網膜と背あわせであった。視神経近傍で作られた神経網膜の神経線維は、視神経を通り中枢へ投射していた。この再生過程は孵化直前でも起こったが、網膜の層構造はやや不完全であった。

En(s)-Pax6delC+導入では眼球および網膜はほとんど形成されず、３種の変異体 F258S、R26G、R128C では不完全な層構造の網膜が形成された。したがって、今回得られた再生網膜は実験操作の人工産物ではなく、Pax6 遺伝子固有の機能によることが示された。さらに、Pax6 変異体のうち、paired domain の変異体 R26G、R128C の方が、homeodomain の変異体 F258S より網膜の層構造形成が不完全であった。したがって、網膜再生には Pax6 の paired domain が関与していることが示唆された。

マウスでは、同様に色素上皮から神経網膜が再生されたが、層構造が不完全であった。

D. 考察

今回の研究によって、Pax6 遺伝子を用いれば、幼若網膜色素上皮細胞を幹細胞として神経網膜を作成できることが明らかになった。作られた神経網膜の神経線維は、視神経を通り中枢へ投射しており、網膜を再生させて視覚復元につながることが示された。

重要なことに、Pax6 を導入すると、後期までほぼ完全な網膜を作れることが判明した。網膜に分化する能力をもつ幹細胞が成熟した色素上皮細胞内に存在することが明らかになった意義は大きい。

哺乳類では、完全な層構造をもつ網膜の再生にはいまだ成功していない。また、完全な層構造をもつ網膜を作成したとしても、これを傷害された網膜に移植で置き換えることは技術的に困難で

あり、たとえ可能であっても、シナプスが形成して脳の視中枢内のしかるべき視覚領域に神経が投射するとは思えない。

しかし、近年、網膜色素変性症モデルマウスに、かなり未熟な正常マウス胎児網膜をシート状に移植し、視覚の改善がみられたとの実験が報告された。さらに、ヒトで網膜移植を行った症例の報告があり、ここでは、網膜色素変性症成人患者の障害された黄斑部網膜下に胎児網膜を色素上皮ごと移植し、１年後に視力が改善したとある。移植網膜は生着し、拒絶反応や網膜浮腫は見られなかったと言う。これらの網膜移植では、移植されるのはかなり未熟な胎児網膜で、レシピエント網膜内で分化し、シナプスを形成するものと思われる。いずれも網膜色素変性症であるので、視細胞以外の網膜構造はある程度温存されており、視細胞が形成されてシナプスが繋がれば、効果があると思われる。

胎児組織の使用に大きな制限があるので、今回の我々の研究で得られた再生網膜がこのような治療に応用されることは大きく期待できる。哺乳類でいまだ不完全な網膜組織が得られていないが、完全な層構造を必要とせず幼若な組織でもよい点も有利である。

E．結論

眼形成遺伝子Pax6を鶏網膜色素上皮細胞に導入して、ほぼ完全な層構造をもつ神経網膜を作ることに成功した。哺乳類でも不完全ながら網膜組織を形成できた、本研究は、網膜を再生、移植して視覚を復元する治療に応用が期待できる。

F．健康危険情報

該当する危険は無し

G．研究発表

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H. 知的財産権の出願・登録状況

1　特許申請

色覚不全動物の色覚復元方法

(出願番号2001-168376)

発明者：東　範行、半田　宏、実験動物中央研究所

出願人：東　範行、半田　宏、実験動物中央研究所

2　実用新案登録　　なし

3　その他　　なし

研究成果の刊行に関する一覧表

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Kawase E, Nishina S, Kumagai K, Azuma N.	Infantile case for occlusive microvascular retinopathy after bone marrow transplantation.	Jpn J Ophthalmol	49	318-320	2005
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Clinical Report

Microdeletion in the *SHOX* 3′ Region Associated With Skeletal Phenotypes of Langer Mesomelic Dysplasia in a 45,X/46,X,r(X) Infant and Leri–Weill Dyschondrosteosis in her 46,XX Mother: Implication for the *SHOX* Enhancer

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It is known that *SHOX* nullizygosity results in Langer mesomelic dysplasia (LMD) and *SHOX* haploinsufficiency leads to Leri–Weill dyschondrosteosis (LWDC). Here, we report on a microdeletion in the *SHOX* 3′ region identified in a Japanese infant with LMD-compatible skeletal features and a 45,X[191]/46,X,r(X)(p22.3q24)[9] karyotype and in her mother with LWDC-compatible skeletal features and a normal 46,XX karyotype. Physical and auxological examinations revealed mesomelic appearance, ulnarly deviated hands, and borderline micrognathia in the infant, and relatively short forearms and lower legs in the mother. Radiological studies indicated mesomelia, markedly curved radii, hypoplastic ulnas and fibulas, and metaphyseal splaying in the infant, and borderline to mild curvature of the radii, decreased carpal angles, and high-normal triangularization index in the mother. Cytogenetic and molecular studies showed that the ring X chromosome of the infant was missing *SHOX* and of paternal origin, whereas the cytogenetically normal X chromosomes of the infant and one of the two X chromosomes of the mother, though they retained *SHOX* with normal coding sequences, had a microdeletion in the *SHOX* 3′ region. The microdeletion started from a position ~200 kb from *SHOX* coding sequences, and spanned 240–350 kb in physical length involving *DXYS233*. The results, in conjunction with those reported by Flanagan et al. [2002], suggest that a *cis*-acting enhancer exists in the *SHOX* 3′ region around *DXYS233*. © 2005 Wiley-Liss, Inc.

KEY WORDS: ***SHOX*; Langer mesomelic dysplasia; Leri–Weill dyschondrosteosis; enhancer; 3′ deletion**

INTRODUCTION

Short stature homeobox containing gene (*SHOX*) cloned from the short arm pseudoautosomal region of the X and the Y chromosome is a transcription factor gene exclusively expressed in the developing skeletal tissues of distal limbs and pharyngeal arches [Rao et al., 1997; Clement-Jones et al., 2000]. *SHOX* haploinsufficiency results in short stature, Turner skeletal features, and Leri–Weill dyschondrosteosis (LWDC) [Ogata, 2002], and *SHOX* nullizygosity leads to Langer mesomelic dysplasia (LMD) [Ogata et al., 2002]. However, ~20% of patients with LWDC have no abnormality in the *SHOX* coding sequences [Ogata, 2002], and two sib patients with LMD have a single normal *SHOX* coding sequence [Zinn et al., 2002]. In this context, Flanagan et al. [2002] have described a large family in which seven patients with two *SHOX* genes accompanied by intact coding sequences have an association of LWDC phenotype with apparent hemizygosity for a region encompassing *DXYS233* at a 3′ position ~300 kb from *SHOX*. Although the results of *DXYS233* microsatellite analysis might be due to amplification failure caused by a polymorphism in the sequence for the primer hybridization, they also demonstrated a monoallelic *SHOX* expression in the bone marrow fibroblasts taken from the distal radius of the proband. Thus, the results suggest that an enhancer for *SHOX* expression exists in a region around *DXYS233*, and that loss of the enhancer results in the development of LWDC.

Here, we report on an infant with LMD-compatible skeletal phenotype and a mosaic ring X chromosome and her mother with LWDC-compatible skeletal phenotype and a normal karyotype. Molecular studies showed a microdeletion in the *SHOX* 3′ region on the cytogenetically normal X chromosome transmitted from the mother to the infant, providing further support for the presence of a *SHOX* enhancer in this region.

CLINICAL REPORT

This Japanese female infant was born at 39 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 50.0 cm (+0.8 SD) and weight 3.73 kg (+2.2 SD). At 7 months of age, she was referred to us because of mesomelic appearance, ulnarly deviated hands, and borderline micrognathia (Fig. 1A). Except for the borderline micrognathia, she had no Turner somatic stigmata such as cubitus valgus, short metacarpals, webbed neck, ear abnormalities, or ptosis. Bone survey showed LMD-compatible skeletal findings such as severely shortened distal limb bones, markedly curved radii, hypoplastic ulnas and fibulas, and metaphyseal splaying (Fig. 1B). Ultrasound studies delineated no cardiac or renal abnormalities. On the latest examination at 1.5 years, her

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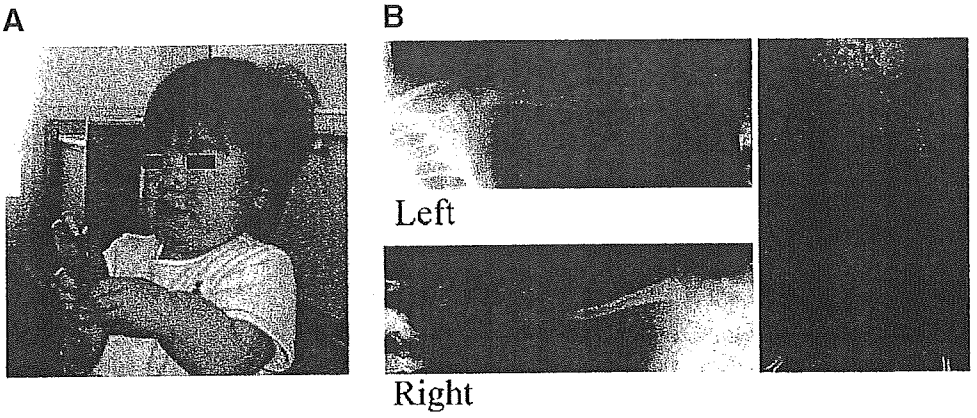

Fig. 1. A: Photograph of the infant at 1.5 years of age. B: Roentgenograms of the infant at 7 months of age.

developmental milestones were normal, and her length was 76.2 cm (−1.8 SD), weight 10.8 kg (+0.5 SD), head circumference 45.7 cm (−0.4 SD), sitting height 49.0 cm (no reference data), and arm span 46.0 cm (no reference data).

The 30-year-old mother was initially regarded as having an apparently normal phenotype with no mesomelic appearance, wrist deformity, or Turner skeletal features. She was 158.1 cm in height (±0 SD) and 53.2 kg in weight (±0 SD), and had no muscular hypertrophy. However, her wrist movement appeared to be somewhat limited. Thus, auxological studies were performed as described by Cameron [1987], revealing relatively short forearms and lower legs, as well as large hands (Fig. 2A). Furthermore, radiological studies of the hands and forearms showed borderline to mild radial curvature, decreased carpal angles (right 101°, left 106°) (normal range, >118°) [Kosowics, 1965], and high-normal triangularization index (right 3.4, left 3.6) (normal range, 1.8–3.7) [Binder et al., 2001] (Fig. 2-B). Thus, she was assessed as having mild LWDC-compatible skeletal phenotype. Allegedly, her 63-year-old father was 162 cm tall (−0.6 SD for his age), her 60-year-old mother 163 cm tall (+1.8 SD for her age), and her 33-year-old sister 158.0 cm tall (±0 SD), with no obviously abnormal

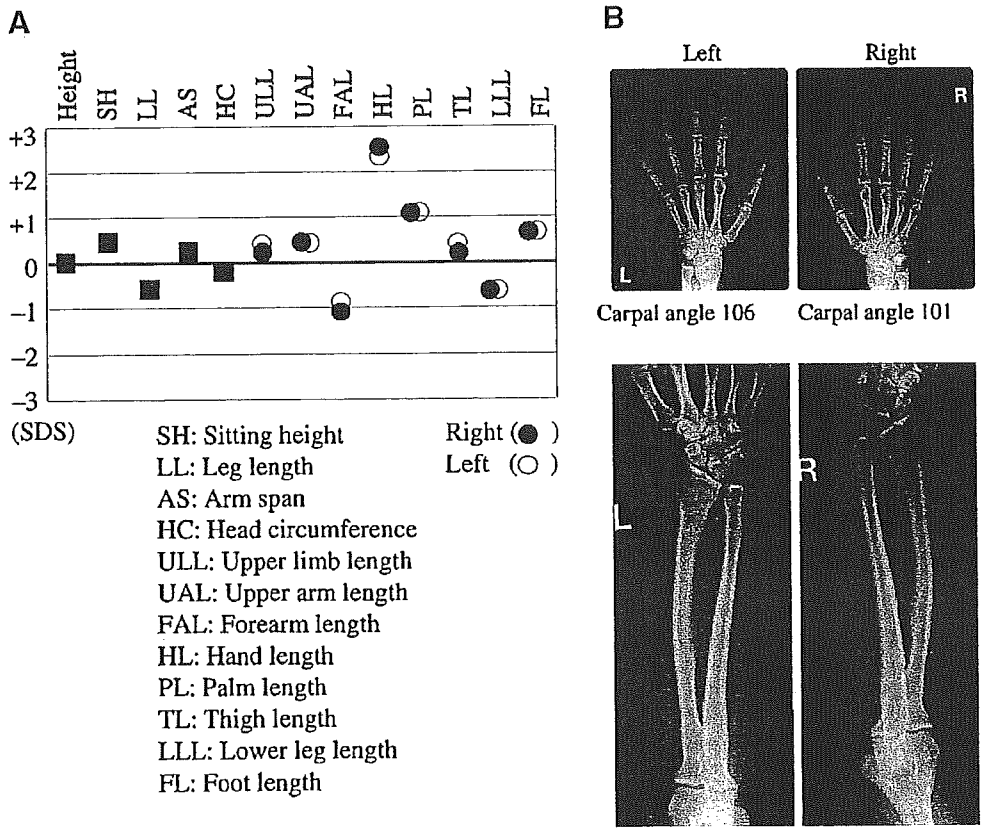

Fig. 2. A: auxological data of the mother. Each measurement is based on the methods described by Cameron [1987], and the auxological data have been assessed by the Japanese body size data (1992–1994) (Research Institute of Human Engineering for Quality of Life, http://www.hql.jp). B: Roentgenograms of the mother at 30 years of age.

findings. Since her parents and sister lived in a local city far away from Tokyo, detailed examinations including auxological and radiological studies were not performed. The 35-year-old father was 178.3 cm in height (+1.4 SD), and clinically normal.

CYTOGENETIC AND MOLECULAR STUDIES

This study has been approved by the Institutional Review Board Committee at National Center for Child Health and Development. After taking written informed consent, peripheral blood samples were obtained from the infant and the parents.

G-banding chromosome analysis was carried out for peripheral lymphocytes, showing a 45,X[191]/46,X,r(X)(p22.3q24)[9] karyotype in the infant, a 46,XX[50] karyotype in the mother, and a 46,XY[50] karyotype in the father. Fluorescence *in situ* hybridization (FISH) analysis was performed for *SHOX* and other four loci as described previously [Ogata et al., 2001a] using *DXZ1* as an internal signal control, demonstrating loss of *SHOX* from the ring X chromosome and determining the breakpoints between *DAX1* and *KAL1* and between *GRIA3* and *GPC3* (Fig. 3A,B). *SHOX* was normally present on the cytogenetically normal X chromosome of the infant and on the two X chromosomes of the mother, as well as on the X and the Y chromosomes of the father. Furthermore, microsatellite analysis was carried out for *SHOX*-5′UTR-CA as described previously [Belin et al., 1998], indicating the paternal origin of the ring X chromosome: the PCR product size was 151 bp in the infant and the mother and 141 and 153 bp in the father. Direct sequencing was performed for *SHOX* coding exons and their flanking introns by the previously described method [Shears et al., 1998], showing no mutation in the infant and the parents.

Thus, a possible deletion at the *SHOX* 3′ region around *DXYS233* was examined for the cytogenetically normal X chromosome of the infant and the mother. PCR deletion analysis was carried out for eight loci/regions with primers reported in Genome Database (http://www.gdb.org/) or designed by us (available on request) using *SHOX* exon 2 as an internal control, identifying a 240–350 kb deletion with the breakpoints between *rs5946324* and *rs5988437* and between *rs4468091* and *RH65317* in the infant (Fig. 3A,C). Furthermore, FISH analysis was carried out with an RP11-309M23 probe (Ensemble Database, http://www.ensembl.org/) that defines a chromosomal region deleted in this infant, showing no signal in the infant and only a single signal in the mother (Fig. 3A,B). The results of the father were normal.

After consultation, the mother decided not to inform the maternal parents and the sister of the results. Thus, together with their residence apart from Tokyo, it was impossible to determine whether the microdeletion was produced as a *de novo* event or transmitted from either of the maternal

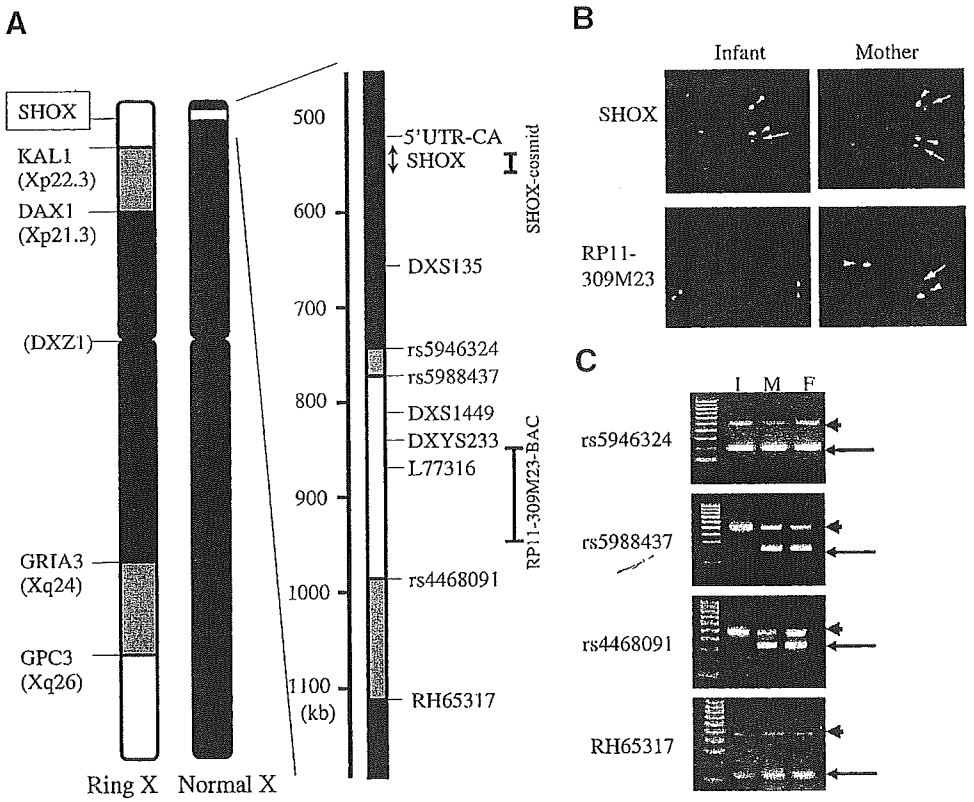

Fig. 3. **A**: Summary of the molecular studies. The black and the white areas denote the preserved and the deleted regions, respectively. The gray areas depict the regions where the breakpoints should exist. For the ring X chromosome, the deleted regions have been determined by FISH analysis for the five loci shown in the left side. For the cytogenetically normal X chromosome, the microdeletion has been demonstrated by PCR deletion analysis for eight loci/regions shown on the right side. The two vertical bars indicate the regions defined by FISH analysis with a SHOX-cosmid probe and an RP11-309M23-BAC probe, respectively. The Arabic numbers represent the physical length from the Xp/Yp telomere (kb). **B**: Representative results of the FISH analysis. The arrows indicate the *SHOX* or the RP11-309M23 region, and the arrowheads represent *DXZ1*. *SHOX* is deleted from the ring X chromosome of the infant, and is normally preserved on the cytogenetically normal X chromosome of the infant as well as on the maternal X chromosomes. The RP11-309M23 region is lost from the cytogenetically normal X chromosome as well as from the ring X chromosome of the infant, and from one of the two X chromosomes of the mother. **C**: Representative results of the PCR deletion analysis. I: infant; M: mother; and F: father. In each PCR analysis, the target sequence (arrows) and the *SHOX* exon 2 (arrowheads) have been amplified concomitantly. The cytogenetically normal X chromosome of the infant is positive for *rs5946324* and *RH65317* and negative for *rs5988437* and *rs4468091*.

parents, especially from the maternal father with a low-normal height, and whether the microdeletion was present or absent in the maternal sister with an average height.

DISCUSSION

The present study showed LMD-compatible skeletal phenotype in an infant with a mosaic ring X chromosome and mild LWDC-compatible skeletal phenotype in her mother with a normal karyotype. Furthermore, it was shown that the ring X chromosome of the infant was missing *SHOX* and of paternal origin, and that the cytogenetically normal X chromosomes of the infant and one of the two X chromosomes of the mother, though they retained *SHOX* with normal coding sequences, had a 240–350 kb microdeletion in the *SHOX* 3′ region.

The results would provide further support for the presence of a *cis*-acting enhancer for *SHOX* in the 3′ region of the gene. In this regard, it is notable that the microdeletion of the infant and the mother encompasses *DXYS233*, because one allele of *DXYS233* was not amplified in the seven familial LWDC patients with two *SHOX* genes accompanied by intact coding sequences described by Flanagan et al. [2002]. Thus, although loss of *DXYS233* was not directly demonstrated in the seven patients, it is inferred that a microdeletion encompassing *DXYS233* also exists in the seven patients, and that the putative enhancer resides in an overlapping deleted region around *DXYS233*. In support of the presence of such an enhancer at a position 200–550 kb from *SHOX* coding sequences, it has been reported in several genes that a deletion or a disruption in a 5′ or 3′ region at a position 10–1,000 kb from the gene coding sequences can affect the function of the corresponding gene [Kleinjan and van Heyningen, 1998]. In this context, two sib patients with typical LMD have apparently one intact allele of *SHOX* (Table I, cases 15 and 16), and ~20% of LWDC patients have no demonstrable *SHOX* abnormalities [Ogata, 2002]. Such patients would be worth analyzing for the putative enhancer region around *DXYS233*.

Clinical features appear to be mild in the infant and the mother. First, the statural growth was well preserved in the infant and the mother. Although the fairly sustained statural growth of the infant and the mother would partly be contributed by the high genetic growth potential as suggested by the relative tall heights of the father and the maternal mother, the previously reported LMD patients with *SHOX* abnormalities have extreme short stature (Table I), and Japanese female patients with *SHOX* haploinsufficiency and normal ovarian function have severe short stature (-2.6 ± 0.8 SD) [Fukami et al., 2004]. Second, while LMD of the infant would be fairly typical, LWDC of the mother was obviously mild as compared with that of adult females with *SHOX* haploinsufficiency [Ogata, 2002; Munns et al., 2003]. In this regard, clinical spectrum in patients with LMD appears to be somewhat variable in terms of the presence or absence of common Turner skeletal features (Table I), and that in patients with *SHOX* haploinsufficiency is known to be variable from borderline short stature only phenotype to severe LWDC phenotype with and without common Turner skeletal features [Ogata, 2002; Munns et al., 2003]. Furthermore, clinical phenotype in patients with a deletion or a disruption affecting a *cis*-acting enhancer for several disease genes has also been reported to range widely from apparently normal phenotype to typical disease phenotype [Kleinjan and van Heyningen, 1998]. Thus, it remains to be elucidated whether the apparently mild phenotypes of the infant and the mother are characteristic of the deletion of the putative enhancer for *SHOX*.

For the skeletal features of the infant and the mother, other possibilities remain tenable at present. First, a hidden

TABLE I. Summary of Patients With Langer Mesomelic Dysplasia

Patients			Clinical features		Mutations			
Case	Age	Sex	Height SDS	Other features[a]	Patient	Father	Mother	Reference
1	Fetus	F	—	N.D.	Deletion/Deletion	(−)/(−)	Deletion/(−)	Belin et al. [1998]
2	Fetus	N.E.	—	N.D.	Deletion/Deletion	Deletion/(−)	Deletion/(−)	Shears et al. [1998]
3	12 years	M	−7.2	None	Deletion/Deletion	Deletion/(−)	Deletion/(−)	Robertson et al. [2000]
4	84 years	F	−5.5	HP, S4M, CV	R153C/V163F	N.E.	N.E.	Zinn et al. [2002]
5	7 years	F	−5.5	HP, CV	Deletion/ R118fsX130	R118fsX130/(−)	Deletion/(−)	Zinn et al. [2002]
6	65 years	M	−7.2	HP, CV	P243fsX321 (homo/hemi)[b]	N.E.	N.E.	Zinn et al. [2002]
7[c]	24 years	F	−8.9	N.D.	R173C/R173C	N.E.	R173C/(−)	Shears et al. [2002]
8[c]	Neonate	M	—	MG?	R173C/R173C	R173C/(−)	R173C/R173C	Shears et al. [2002]
9	1.5 years	M	−4.2	None	Deletion/R168W	Deletion/(−)	R168W/(−)	Ogata et al. [2002]
10	Fetus	F	—	None	Deletion/Deletion	(−)/(−)	Deletion/(−)	Thomas et al. [2004]
11[d]	Adult	M	N.D.	N.D.	A170P/A170P	A170P/(−)	A170P/(−)	Sabherwal et al. [2005]
12[d]	38 years	F	−8.5	N.D.	A170P/A170P	A170P/(−)	A170P/(−)	Sabherwal et al. [2005]
13[d]	Adult	F	N.D.	N.D.	A170P/A170P	A170P/(−)	A170P/(−)	Sabherwal et al. [2005]
14[d]	2 years	F	N.D.	N.D.	A170P/A170P	A170P/(−)	A170P/A170P	Sabherwal et al. [2005]
15[e]	35 years	F	−6.8	HP, CV	Deletion/(−)	N.E.	N.E.	Zinn et al. [2002]
16[e]	33 years	M	−6.2	HP, S4M, CV	Deletion/(−)	N.E.	N.E.	Zinn et al. [2002]
17	1.5 years	F	−1.8	MG?	Deletion/ 3′ deletion	(−)/(−)	3′ deletion/(−)	This report

The (−) symbol indicates the absence of a recognizable mutation. SDS, standard deviation score; F, female; M, male; N.E., not examined; N.D., not described; HP, high arched palate; S4M, short 4th metacarpals; CV, cubitus valgus; and MG, micrognathia.

[a]Except for severe mesomelia and skeletal deformities in the forearms and shanks.

[b]Case 6 is homozygous or homozygous for P243fsX321.

[c]Case 7 is the mother of case 8, and her consanguineous husband has Leri–Weill dyschondrosteosis and the same SHOX mutation.

[d]Cases 11, 12, and 13 are siblings; case 13 is the mother of case 14, and her consanguineous husband has Leri–Weill dyschondrosteosis and the same SHOX mutation.

[e]Case 15 and case 16 are siblings.

mutation might exist in the unexamined regions such as the intron or the promoter sequences of *SHOX* on the cytogenetically normal X chromosome transmitted from the mother to the infant. Second, a different gene(s) involved in the skeletal development of distal limbs might be affected in the infant and the mother. In this regard, Ventruto et al. [1983] have reported a two-generation family in which LWDC-like phenotype is cosegregated with t(2;8)(q31;p21), and the translocation breakpoint has been located at a position ~60 kb telomeric to *HOXD* gene cluster [Spitz et al., 2002]. Furthermore, Kantaputra et al. [1992] have described a large family with an autosomal dominant form of mesomelic dysplasia, and the gene has been mapped to a 2q24–32 region encompassing *HOXD* gene cluster [Fujimoto et al., 1998]. However, loss of *HOXD9–HOXD13* expressed in the limb region has been reported to cause synpolydactyly rather than mesomelic dysplasia [Goodman et al., 2002], so that the relevance of *HOXD* genes to the development of mesomelic dysplasia remains to be clarified, as well as a possible role of the putative global control region for the expression of multiple *HOXD* and other neighboring genes [Spitz et al., 2003].

Further two matters would also be worth pointing out in the present study. First, except for the borderline micrognathia, the infant had no Turner somatic or visceral features in the presence of the mosaic ring X chromosome. This would not be surprising, because clinical features are highly variable in Turner syndrome [Ogata and Matsuo, 1995]. In addition, the ring X chromosome should retain the putative lymphogenic gene(s) between *DMD* and *MAOA* responsible for the development of soft tissue and visceral features [Ogata et al., 2001b], and should be present more frequently in the slowly dividing target tissues for the soft tissue and visceral features than in the rapidly dividing lymphocytes utilized for the karyotype analysis. This would also be relevant to the lack of such features in this infant. Second, the mother had relative large hands. Since this phenotype has also been reported in Turner patients [Gravholt and Weis Naeraa, 1997], it may be characteristic of *SHOX* haploinsufficiency.

In summary, the results, in conjunction with those reported by Flanagan et al. [2002], argue for the presence of a *cis*-acting enhancer in the *SHOX* 3′ region around *DXYS233*. Further studies will permit a definite conclusion on this matter.

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Segmental and Full Paternal Isodisomy for Chromosome 14 in Three Patients: Narrowing the Critical Region and Implication for the Clinical Features

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We report on segmental and full paternal isodisomy for chromosome 14 in three previously unreported Japanese patients. Patient 1 was a 5$^{6}/_{12}$-year-old girl, Patient 2 was a male neonate, and Patient 3 was a 6$^{7}/_{12}$-year-old girl. Physical examination at birth showed various somatic features characteristic of paternal uniparental disomy for chromosome 14 (upd(14)pat) such as hairy forehead, protruding philtrum, micrognathia, small thorax, and abdominal wall defects in Patients 1–3, and the constellation of somatic features was persistently observed in Patients 1 and 3. Radiological studies at birth delineated unique bell-shaped thorax with coat-hanger appearance of the ribs in Patients 1–3, but the thoracic deformity ameliorated in Patients 1 and 3 by mid childhood. Chromosome analysis showed a 46,XX karyotype in Patients 1 and 3 and was not performed in Patient 2. Microsatellite analysis indicated full paternal isodisomy for chromosome 14 in Patients 1 and 2 and segmental paternal isodisomy for chromosome 14 distal to *D14S981* at 14q23.3 in Patient 3. Methylation specific PCR assay for the differentially methylated region (DMR) of *GTL2* at 14q32 yielded positive products with methylated allele specific primers and no products with unmethylated allele specific primers in Patients 1–3. Since clinical phenotype was similar between Patient 3 with segmental upd(14)pat and Patients 1 and 2 with full upd(14)pat, the results are keeping with the 14q32 localized imprinted genes as the critical components of the phenotype observed in upd(14)pat and help narrow the search for additional genes to the ~40 Mb region distal to *D14S981*. Furthermore, it is likely that the characteristic thoracic deformity ameliorates with age.

KEY WORDS: paternal disomy; segmental disomy; chromosome 14; somatic features; thoracic deformity

INTRODUCTION

Paternal uniparental disomy for chromosome 14 (upd(14)pat) is associated with a distinctive constellation of clinical features such as characteristic face with blepharophimosis, prominent philtrum and micrognathia, small thorax, abdominal wall defects, and developmental retardation [Sutton and Shaffer, 2000; Chu et al., 2004]. This condition is also radiologically characterized by unique bell-shaped thorax with coat-hanger appearance of the ribs [Offiah et al., 2003]. The thoracic deformity often results in lethal respiratory failure during infancy. In addition, polyhydramnios is seen in most pregnancies with upd(14)pat.

To date, upd(14)pat has been identified in a total of 15 patients [Wang et al., 1991; Papenhausen et al., 1995; Walter et al., 1996; Cotter et al., 1997; Klein et al., 1999; Berend et al., 2000; Yano et al., 2001; Coveler et al., 2002; Kurosawa et al., 2002; McGowan et al., 2002; Offiah et al., 2003; Chu et al., 2004; Stevenson et al., 2004]. Of the 15 patients, 10 patients have Robertsonian translocations involving chromosome 14 such as t(13;14) and t(14;14), and the remaining five patients have a normal karyotype. Genotyping analysis has confirmed upd(14)pat in such patients, including a segmental isodisomy for a 14q12–14qter region in a single patient with characteristic upd(14)pat phenotype [Coveler et al., 2002]. This implies the involvement of an imprinted gene(s) in the development of upd(14)pat phenotype, and locates the major locus or loci for the upd(14)pat phenotype to the 14q12–14qter region. Consistent with this, the 14q32 segment is known to contain imprinted genes such as *DLK1*, *GTL2* (also known as *MEG3*), *PEG11*, and *MEG8* [Charlier et al., 2001; Cavaille et al., 2002]. The possibility that the clinical phenotype in upd(14)pat is contributed by the unmasking of a recessive allele(s) due to isodisomy is unlikely, because apparently full paternal heterodisomy as well as isodisomy has been found in patients with the distinct clinical phenotype [Chu et al., 2004].

However, the critical region is still large. Furthermore, since only the infantile phenotypes have been described in most patients, the long-term clinical course remains to be clarified in this condition. Here, we report three patients with upd(14)pat.

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The results further narrow down the critical region and suggest improvement of thoracic deformity with age.

MATERIALS AND METHODS

Patients

Three previously unreported Japanese patients (one male and two females) were examined in this study. Their clinical features are summarized in Table I. Patients 1–3 were born prematurely after pregnancies complicated by polyhydramnios. Patients 1 and 2 were delivered by Caesarean sections because of placental abnormalities, while Patient 3 was born by vaginal delivery. Patient 1 had severe asphyxia at birth, and was treated with endotracheal intubation and mechanical ventilation for 4 days and with oxygen and nasal directional positive airway pressure for the following 61 days at the neonatal intensive care unit (NICU). Subsequently, oxygen was supplied via a nasal mask for 355 days at the pediatric ward. Patient 2 died of respiratory failure at 2 hr of age despite an intensive care including mechanical ventilation and oxygen. Patient 3 had mild asphyxia at birth, and received oxygen in an incubator for 34 days at the NICU and via nasal mask for the following 96 days at the pediatric ward. After the discharge, Patients 1 and 3 had uneventful clinical course with no episode of respiratory infection. In addition, although pulmonary function test was not performed, they were able to play actively with peers.

Birth size appeared to be well preserved, although gestational age-matched reference data were not available in Patient 1. Childhood body size remained within the normal range in Patients 1 and 3. Moderate developmental retardation was indicated in childhood of Patients 1 and 3. Physical examination at birth revealed various somatic features consistent with upd(14)pat such as hairy forehead, protruding philtrum, micrognathia, small thorax, and abdominal wall defects in Patients 1–3, and the constellation of somatic features persisted into childhood in Patients 1 and 3. Radiological studies at birth delineated unique bell-shaped thorax with coat-hanger appearance of the ribs in Patients 1–3, but the thoracic deformity ameliorated in Patients 1 and 3 by mid childhood (Fig. 1). Parental age was variable at the time of birth of Patients 1–3 and parental heights were normal in Patients 1 and 3.

Conventional and Molecular Cytogenetic Studies

This study has been approved by the Institutional Review Board Committee at National Center for Child Health and Development. Chromosome analysis was performed on 50 peripheral lymphocytes in Patients 1 and 3. Fluorescence *in situ* hybridization (FISH) analysis was also carried out on lymphocyte metaphase spreads in Patients 1 and 3, using a ~186 kb BAC probe containing *DLK1* (RP11-566J3) and a ~165 kb BAC probe containing *GTL2*, *PEG11*, and *MEG8* (RP11-123M6) (BACPAC Resources Center, http://bacpac.chori.org/), together with a 14q telomere probe (Vysis, http://www.vysis.com/) used as an internal signal control. The RP11-566J3 and RP11-123M6 probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the 14q telomere probe was detected according to the manufacture's protocol.

Microsatellite Analysis

Microsatellite analysis was performed for multiple loci on chromosome 14 (Fig. 2A). In brief, leukocyte genomic DNA of Patients 1–3 and their parents was amplified by polymerase chain reaction (PCR) with fluorescently labeled forward primers and unlabeled reverse primers, and the PCR products were determined for the fragment size on an ABI PRISM 310 autosequencer using GeneScan (Applied Biosystems, http://www.appliedbiosystems.com/). The primer sequences were as described in Genome Database (http://www.gdb.org/).

Methylation Specific PCR Assay

Methylation pattern was analyzed for the differentially methylated region (DMR) of *GTL2* where cytosines at CpG dinucleotides are methylated on the paternally derived allele and unmethylated on the maternally derived allele [Murphy et al., 2003]. In short, after bisulphite treatment with EZ DNA methylation kit (Zymo Research, http://www.zymor.com/) that converts all the cytosines except for methylated cytosines at the CpG islands into uracils and subsequently thymines, PCR amplification was performed with a pair of primers specific to the methylated allele of paternal origin and with another pair of primers specific to the unmethylated allele of maternal origin. The primer sequences and the PCR conditions were as reported previously [Murphy et al., 2003]. For a control, a DNA sample from a clinically normal individual was utilized with permission.

RESULTS

Conventional and Molecular Cytogenetic Studies

Normal female karyotype was identified in Patients 1 and 3, and two signals were detected by RP11-566J3 and RP11-123M6 as well as by the 14q telomere probe in Patients 1 and 3. Thus, the karyotype was determined as 46,XX.ish 14q32(DLK1 × 2,GTL2 × 2,PEG11 × 2,MEG8 × 2) in Patients 1 and 3.

Microsatellite Analysis

The data are summarized in Figure 2A and representative results are shown in Figure 2B. In Patients 1 and 2, single peaks only were detected for all the loci examined, and paternal isodisomy was demonstrated for multiple loci on the various parts of chromosome 14. In Patient 3, single peaks only were identified for *D14S80* and 20 loci from *D14S1069* to *D14S1007*, and paternal isodisomy was confirmed for five loci (*D14S1000*, *D14S267*, *D14S985*, *D14S1010*, and *D14S292*) on the distal part of 14q. By contrast, two peaks were found for 11 loci from *D14S1021* to *D14S981*, and biparental origin was demonstrated for seven loci (*D14S608*, five loci from *D14S75* to *D14S1026*, and *D14S1046*) on the middle to proximal part of 14q. Furthermore, the genotyping results of the remaining four loci (*D14S1021*, *D14S121*, *D14S271*, and *D14S981*) were also consistent with biparental origin, although paternal heterodisomy for *D14S1021* and *D14S981*, and maternal heterodisomy for *D14S121* and *D14S271* might theoretically be possible. Taken together, the results indicated segmental paternal isodisomy for chromosome 14 distal to *D14S981* at 14q23.3 in Patient 3.

Methylation Specific PCR Assay

Although PCR products were obtained with the methylated allele specific primers, no PCR products were yielded with the unmethylated allele specific primers in Patients 1–3 (Fig. 3). In a control subject, PCR products were obtained with both of the primers.

DISCUSSION

The results indicate that Patients 1 and 2 had full paternal isodisomy and Patient 3 had segmental paternal isodisomy for chromosome 14. The full paternal isodisomy in Patient 1 with a normal karyotype could most likely be due to duplication of

TABLE I. Summary of Patients with Paternal Uniparental Disomy for Chromosome 14

	This study			Reported patients	
	Patient 1	Patient 2	Patient 3	Full disomy	Seg. disomy
Age at description	5 6/12 years	Deceased (at 2 hr of age)	6 7/12 years	Fetus–9 years (n = 14)[c]	Not described (n = 1)[d]
Sex	Female	Male	Female	M:F = 4:9[e]	Male
Disomy region	Full	Full	14q23.3–qter	Full	14q12–qter
Disomy pattern	Isodisomy	Isodisomy	Isodisomy	I:H:M = 7:4:2[f]	Isodisomy
Polyhydramnios	+	+	+	12/12	+
Placental abnormality	+ (abruption)	+ (forelying)	–	1/1 (large)	+ (abruption)
Delivery	Caesarean	Caesarean	Vaginal	V:C = 4:3[g]	Caesarean
Premature delivery	+ (32 weeks)	+ (34 weeks)	+ (36 weeks)	8/9	+ (29 weeks)
APS at 1 and 5 min	1 and 6	1 and 6	6 and 7	4/6[h]	N.D.
Birth length	41.0 cm	45.7 cm (+0.6 SD)[a]	46.0 cm (−0.3 SD)[a]	...	25 centile
Birth weight	1.85 kg	3.17 kg (+2.5 SD)[a]	2.94 kg (+1.2 SD)[a]	...	95 centile
Birth HC	30.6 cm	35.5 cm (+2.5 SD)[a]	29.4 cm (−2.3 SD)[a]	...	80 centile
IUGR	...	–	–	1/7	–
Present height	102.1 cm (−1.7 SD)[b]	...	121.3 cm (+1.0 SD)[b]	...	N.D.
Present weight	15.1 kg (−1.4 SD)[b]	...	25.8 kg (+1.4 SD)[b]	...	N.D.
Present HC	51.1 cm (+0.4 SD)[b]	...	52.0 cm (+0.6 SD)[b]	...	N.D.
Growth failure	–	...	–	3/3	N.D.
Developmental retardation	+ (DQ = 50)	...	+ (DQ = 52)	6/6	+
Seizure	–	...	–	2/3	–
Frontal bossing	+	–	–	1/1	+
Hairy forehead	+	+	+	3/4	+
Blepharophimosis	+	–	+	9/9	+
Depressed nasal bridge	+	+	+	7/7	+
Anteverted nares	+	+	–	2/4	–
Small ears	±	+	–	6/6	+
Feeding difficulty	+	...	+	5/5	+
Protruding philtrum	+	+	+	8/8	+
Puckered lips	–	–	–	2/4	–
Micrognathia/retrognathia	+	+	+	5/6	+
Short/webbed neck	+	+	+	7/7	+
Laryngomalacia	–	...	+	1/1	N.D.
Small thorax	+	+	+	10/10	+
Mechanical ventilation	+	+	–	9/10	+
Lethal respiratory failure	–	+	–	4/13	–
Congenital heart disease	–	+ (ASD)	–	3/4[i]	ASD, VSD, PS
Abdominal wall defect	+ (diastasis recti)	+ (omphalocele)	+ (diastasis recti)	10/10[j]	+ (omphalocele)
Inguinal hernia	–	–	–	1/1	+
Kyphoscoliosis	+	–	+	1/1	+
Coxa valga	+	...	–	1/1	+
Short limbs	–	–	–	6/8	+
Long fingers	–	–	–	3/3	–
Joint contractures	+	–	+	6/7	–
Paternal age at birth	36	N.E.	24	...	34
Maternal age at birth	36	42	27	...	33
Paternal height	178 cm (+1.6 SD)[b]	N.E.	171 cm (+0.1 SD)[b]	...	...
Maternal height	153 cm (−1.0 SD)[b]	N.E.	155 cm (−0.6 SD)[b]	...	...

APS, Apgar score; HC, head circumference; IUGR, intrauterine growth retardation; SD, standard deviation; DQ, developmental quotient; ASD, atrial septal defect; VSD, ventricular septal defect; PS, pulmonary stenosis; N.E., not examined; and N.D., not described.

[a]Assessed by the gestational age- and sex-matched Japanese reference data from the Ministry of Health, Labor, and Welfare (http://wwwdbtk.mhlw.go.jp/toukei/); no data are available for neonates born at 32 weeks of gestational age.

[b]Assessed by the age- and sex-matched Japanese reference data [Suwa et al., 1992].

[c]Wang et al. [1991]; Papenhausen et al. [1995]; Walter et al. [1996]; Cotter et al. [1997]; Klein et al. [1999]; Berend et al. [2000]; Yano et al. [2001]; Kurosawa et al. [2002]; McGowan et al. [2002]; Offiah et al. [2003]; Chu et al. [2004]; and Stevenson et al. [2004].

[d]Coveler et al. [2002].

[e]Male: Female = 4:9; the sex is not described in one of the two patients reported by Berend et al. [2000].

[f]Isodisomy:Heterodisomy:Mixture of isodisomy and heterodisomy = 7:4:2; the type of disomy is unreported in the patient described by Offiah et al. [2003].

[g]Vaginal:Caesarean = 4:3; the delivery type is unreported in the remaining patients.

[h]APS at 1 min $\leq$ 3.

[i]ASD, VSD, and aortic anomaly are manifested by three different patients, respectively.

[j]All the 10 patients have diastasis recti, and omphalocele is absent in the 10 patients.

In the column summarizing the clinical features in 14 patients with full disomy reported in the literature, the denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature; thus, the differences between the denominators and the numerators denote the number of patients evaluated to be negative for that feature.

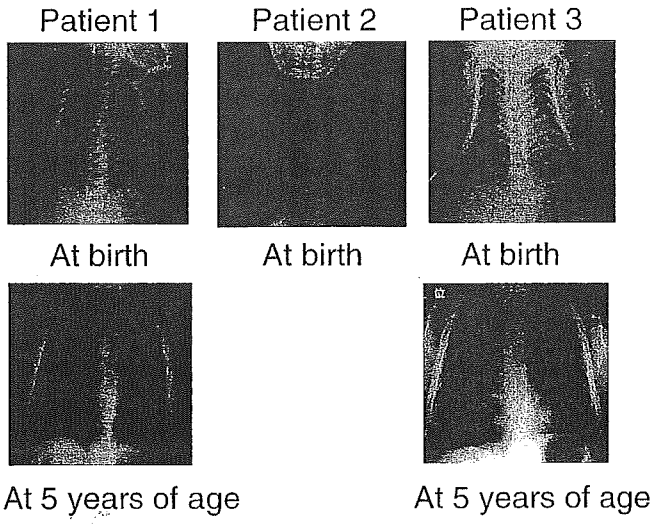
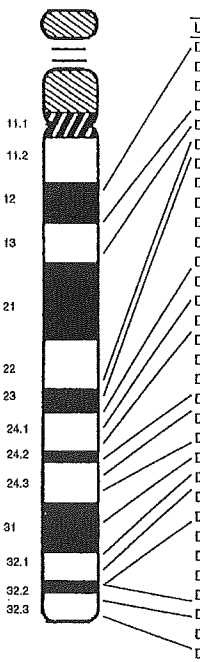


Fig. 1. Roentgenograms of Patients 1–3. The unique bell-shaped thorax with an arched appearance of the ribs is obvious in Patients 1–3 at birth, and the thoracic deformity has ameliorated in Patients 1 and 3 at 5 years of age.

chromosome 14 from a monosomic zygote (monosomy rescue), although loss of maternally derived chromosome 14 from a trisomic zygote (trisomy rescue) or fertilization between a disomic gamete and a nullisomic gamete (gamete complementation) with no preceding recombination at meiosis I might also be possible [Robinson, 2000]. This mechanism could also be applied to Patient 2. However, since karyotype was not examined in Patient 2, it is also possible that Patient 2 had a paternally derived chromosomal abnormality such as i(14q) often associated with upd(14)pat [Kotzot, 2001]. The segmental paternal isodisomy in Patient 3 with a normal karyotype would be explained by assuming mitotic recombination in the first zygotic division or in a very early postzygotic division, followed by loss of the normal cell lineages and reciprocal maternal disomic cell lineage (post-fertilization errors) [Robinson, 2000], although a more complex mechanism assuming errors in both meiosis I and mitosis (another form of trisomy rescues) might also be possible [Kotzot, 2001]. Furthermore, the results of methylation specific PCR assay are consistent with upd(14)pat for the examined DMR of *GTL2* in Patients 1–3.

Genotype–phenotype correlations in Patients 1–3, in conjunction with those in the previously described patients, are informative for the localization of the major locus or loci relevant to the development of the upd(14)pat phenotype (Table I). Patient 3 with segmental paternal isodisomy had a constellation of clinical features characteristic of upd(14)pat at birth, as did Patients 1 and 2 with full paternal isodisomy for chromosome 14. In addition, the clinical phenotype was comparable between Patients 1 and 3 in childhood. Furthermore, although Patient 2 had lethal respiratory failure, atrial septal defect, and omphalocele, such severe features were absent in Patient 1 with full paternal isodisomy as well as in Patient 3 with segmental paternal isodisomy. Similarly, although the previously described patient with segmental isodisomy for 14q12–qter [Coveler et al., 2002] had cardiovascular lesion and omphalocele that were absent in Patient 3 with segmental isodisomy for 14q23.3–qter, cardiovascular lesion is not a consistent feature even in patients with full upd(14)pat, and omphalocele is absent in patients with full upd(14)pat

A



	Patient 1				Patient 2				Patient 3			
Locus	Mother	Patient	Father	Assesment	Mother	Patient	Father	Assesment	Mother	Patient	Father	Assesment
D14S80	105/109	105	105/105	N.I.	95/105	97	97	upd(14)pat	97/105	97	97/105	N.I.
D14S1021	263	265	263/265	upd(14)pat	263/271	263	263	N.I.	263	261/263	261/263	(Biparental)*
D14S608	196/208	196	196/208	N.I	212/216	204	204	upd(14)pat	216/220	196/220	196/204	Biparental
D14S121	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	141/151	141/151	147/151	(Biparental)†
D14S75	188/194	184	180/184	upd(14)pat	184	186	184/186	upd(14)pat	190/194	184/194	184/192	Biparental
D14S980	163/167	167	165/167	N.I.	168/182	168	168	N.I.	155/167	167/183	163/183	Biparental
D14S1038	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	221/225	219/221	219/225	Biparental
D14S63	206/212	214	206/214	upd(14)pat	202/206	204	202/204	upd(14)pat	202/214	206/214	206	Biparental
D14S1026	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	134/142	124/134	124/132	Biparental
D14S271	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	227/229	227/229	227	(Biparental)†
D14S1046	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	214/216	212/214	212/218	Biparental
D14S981	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	193/197	195/197	195/197	(Biparental)*
D14S1069	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	206/212	212	206/212	N.I.
D14S1065	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	256	256	256/258	N.I.
D14S1011	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	184/186	184	184	N.I.
D14S588	118/126	114	114	upd(14)pat	114/126	114	114/122	N.I.	114/126	114	114/122	N.I.
D14S1029	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	176	176	176	N.I.
D14S258	171/175	169	169/175	upd(14)pat	171/175	169	169/175	upd(14)pat	169	169	169/175	N.I.
D14S1028	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	231/241	231	231	N.I.
D14S1025	145/149	143	143	upd(14)pat	143/145	143	143	N.I.	143	143	143/149	N.I.
D14S1036	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	198/206	206	206/210	N.I.
D14S1000	N.E.	N.E.	N.E.	...	N.E.	N.E.	N.E.	...	124/126	128	122/128	upd(14)pat
D14S81	172/180	172	172/180	N.I.	175/181	181	179/181	N.I.	172/180	180	180	N.I.
D14S617	165	161	161/165	upd(14)pat	137/165	169	143/169	upd(14)pat	137/165	137	137	N.I.
D14S979	199/201	191	191/195	upd(14)pat	196/208	202	202	upd(14)pat	197/203	197	197	N.I.
D14S267	215/217	189	189/197	upd(14)pat	201/211	209	201/209	upd(14)pat	201	209	201/209	upd(14)pat
D14S250	158	160	160/166	upd(14)pat	154/162	160	160/170	upd(14)pat	158/160	158	158	N.I.
D14S1006	126/138	126	126/138	N.I.	126/130	140	126/140	upd(14)pat	136	136	136/140	N.I.
D14S985	132/136	136	128/136	N.I.	131/137	133	133	upd(14)pat	128/132	130	130	upd(14)pat
D14S1010	142/148	144	144/146	upd(14)pat	142	134	134/144	upd(14)pat	134/152	142	142	upd(14)pat
D14S292	106/110	112	108/112	upd(14)pat	106/108	110	110/112	upd(14)pat	110	104	104	upd(14)pat
D14S1007	111/123	111	111	N.I.	111/121	121	121/123	N.I.	111/121	121	121/123	N.I.

B

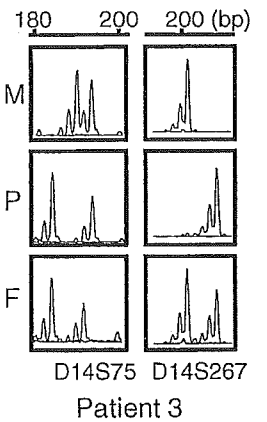


Fig. 2. Microsatellite analysis. **A**: Summary of the results. The ideogram of chromosome 14 is shown with the cytogenetic location of the microsatellite loci examined. The locus order is based on Ensembl Genome Browser (http://www.ensembl.org). The Arabic numbers indicate the PCR product sizes in bp. * Paternal uniparental heterodisomy could theoretically be possible. † Maternal uniparental heterodisomy could theoretically be possible. upd(14)pat, paternal uniparental disomy for chromosome 14; N.E., not examined; and N.I., not informative. **B**: Representative microsatellite results (M, mother; P, patient; and F, father). D14S75: Patient 3 is heterozygous with the paternally and maternally derived peaks, indicating biparental origin of this locus. D14S267: One of the two paternal peaks only is transmitted to Patient 3, indicating paternal uniparental isodisomy for this locus.

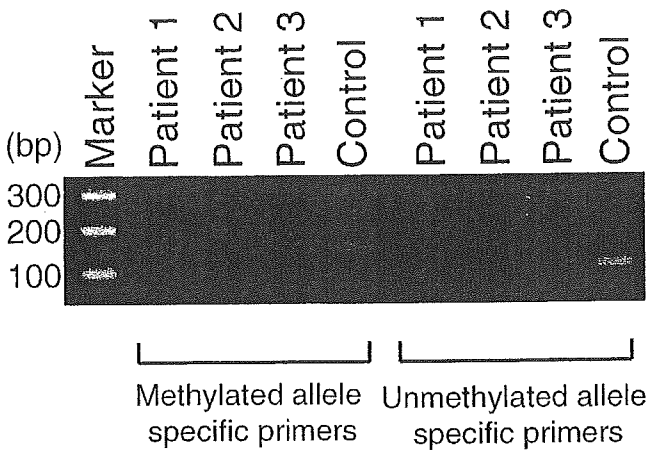


Fig. 3. Methylation specific PCR assay. In patients 1–3, PCR products have been obtained with methylated allele specific primers, but not with unmethylated allele specific primers.

except for Patient 2 in this study. These findings suggest that clinical phenotype in upd(14)pat can be associated with variable expressivity and penetrance; thus, for example, omphalocele and diastasis recti could be regarded as clinical features of different expressivity in a single phenotypic spectrum. It is inferred, therefore, that the isodisomic 14q23.3–14qter segment distal to *D14S981* in Patient 3 is the critical region for the development of upd(14)pat phenotype, and that an imprinted gene with a significant phenotypic effect is absent from the region proximal to 14q23.3. The critical region defined in this study is smaller than that defined previously [Coveler et al., 2002] and spans ~40 Mb in physical length (Ensembl Genome Browser Database, http://www.ensembl.org/).

The present location would be supported by the previous data. First, the 14q32 segment within the critical region is known to harbor both paternally expressed genes such as *DLK1* and *PEG11*, and maternally expressed genes such as *GTL2* and *MEG8* [Charlier et al., 2001; Cavaille et al., 2002]. Second, phenotypic comparisons of paternal or maternal disomy for chromosome 14 with 14q deletions of known parental origin suggest the presence of imprinted genes on a 14q23–q32 region [Sutton and Shaffer, 2000]. Third, the 14q23.3–14qter critical region shares homology with mouse chromosome 12 that contains a cluster of imprinted genes including *Dlk*1 and *Gtl*2 (Jackson Laboratory, http://www.informatics.jax.org/), and murine upd(12)pat leads to skeletal features reminiscent of those observed in human upd(14)pat [Sutton et al., 2003]. These findings would argue for the relevance of an imprinted gene(s) on the critical region to the development of upd(14)pat phenotype.

The present study extends much of the characteristic phenotype through early development. However, the unique bell-shaped thorax with coat-hanger appearance of the ribs, though it was obvious at birth, became non-recognizable by mid-childhood in Patients 1 and 3. Furthermore, the improvement of radiographic findings was apparently associated with that of respiratory function. These findings suggest that thoracic deformity and resultant respiratory distress may ameliorate with age. Thus, the life-prognosis can be expected to be fairly good for the patients who survived the infantile period.

To date, there has been only a single report documenting a patient with upd(14)pat after infancy. Wang et al. [1991] described a 9-year-old girl with characteristic somatic features and mental retardation who had a 13;14 Robertsonian translocation and molecularly confirmed paternal disomy. Although long-term clinical course and radiological findings have not been described, the patient is apparently free from a life-threatening respiratory dysfunction at the time of description. The findings would also support the fairly good life prognosis in survivors.

In summary, the results imply that an imprinted gene(s) for the development of upd(14)pat phenotype is localized to the ~40 Mb segment distal to *D14S981* at 14q23.3, and that the characteristic thoracic deformity may ameliorate with age. Further studies are needed to identify possible additional imprinted loci contributing to the upd(14)pat phenotype and to clarify the natural clinical course of the syndrome.

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Minireview

DHPLC in clinical molecular diagnostic services

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Abstract

A high-capacity low-cost mutation scanning method based on denaturing high-performance liquid chromatography (DHPLC) has been recently introduced. We have implemented an automated and cost-effective strategy using DHPLC. To facilitate the semi-automated analysis of multiple exons, two steps were taken. The first step was the development of a PCR protocol for the amplification of multiple exons under the same conditions. Primer sets, which amplify each exon in the entire gene, were aliquoted to and air-dried on a 96-well format PCR plate. In this way, all the exons in a gene can be simultaneously amplified on a single PCR machine. The second step was the serial DHPLC analysis of multiple amplicons under conditions optimal for each amplicon. We named the 96-well plate containing the primer pairs and the corresponding computer file used to analyze each amplicon under the pre-determined optimal conditions as the "Condition-Oriented-PCR primer-Embedded-Reactor plate," or the COPPER plate. We have developed COPPER plate systems for more than 20 congenital disorders including classic congenital syndromes like Marfan syndrome (*FBN1*: 65 amplicons), CHARGE syndrome (*CHD7*: 39 amplicons), de Lange syndrome (*NIPBL*: 46 amplicons), Sotos syndrome (*NSD1*: 30 amplicons), and Rubinstein–Taybi syndrome (*CREBBP*: 41 amplicons). Using the COPPER plate system, we are functioning as a reference laboratory for the clinical molecular diagnosis of congenital malformation syndromes and are presently analyzing more than 200 samples annually from all over Japan.

Keywords: Mutation analysis; Multiple malformation syndrome; DHPLC; Heteroduplex analysis; Molecular diagnosis

Introduction

As catalogued in pediatric genetics textbooks like "*Metabolic and Molecular Basis of Inherited Diseases*" [1] and "*Inborn Errors of Development*" [2], several hundreds of genes have been shown to cause human congenital disorders. The identification of these causative genes has offered us a wonderful opportunity to delineate the molecular basis of these disorders. Molecular diagnosis offers valuable information to the patients and their families in terms of prognosis, preventing complications, and providing accurate genetic counseling. Thanks to the completion of the human genome project, information on the human genome sequence is now readily accessible to everybody and, theoretically, any gene can be tested by the direct sequencing of PCR products amplified from the patient's genomic DNA. However, identifying pathogenic mutations is difficult when the causative gene has a large number of exons. In such cases, direct sequencing is expensive, technically demanding, and time consuming. Recently, a high-capacity low-cost mutation scanning method based on denaturing high-performance liquid chromatography (DHPLC) has been introduced [3–5]. Over the past six years, we have developed an automated and cost-effective strategy using DHPLC [6–12]. In this review, we would like to share our strategies with the readers of this journal.

DHPLC compares two chromosomes as a mixture of denatured and reannealed PCR amplicons [13]. When the PCR amplicon does not contain a mutation, the sense strand and the anti-sense strand of the PCR amplicons are completely complementary to each other. In this case, the

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