別紙4

Alam MS., Ohsako S., Tay TW., Tsunekawa N., Kanai N., and Kurohmaru M.	Di(n-butyl) phthalate induces vimentin filaments disruption in rat Sertoli cells: A possible relation with spermatogenic cell apoptosis	Anatomia Histologia Embryologia	39	189-193	2010
緒方勤	エストロゲン様内分泌撹乱化学物質と男性性機能低下：疫学データと個体感受性解析	日小児泌会誌	日小児泌会誌	16 (2):	130–135,
緒方勤	男児外陰部異常症および精子形成障害と内分泌撹乱化学物質感受性：エストロゲン受容体a遺伝子のハプロタイプ解析.	医のあゆみ	225 (9) (	954–959,	2009
緒方勤	男児外陰部異常症	臨泌63 (2) (特集：泌尿器科医のための内分泌学ことはじめ)		109–115,	2009
緒方勤	小児疾患と環境化学物質：遺伝—環境相互作用の観点から	科学	岩波書店 79	982–983,	2009
Ohsako S., Fukuzawa N., Ishimura R., Kawakami T., Wu Q., Nagano R., Zaha H., Sone H., Yonemoto J., and Tohyama C.	Comparative contribution of the aryl hydrocarbon receptor gene to perinatal stage development and dioxin-induced toxicity between the urogenital complex and testis in the mouse.	Biol Reprod	82	636-643	2010
Alam MS., Ohsako S., Matsuwaki T., Zhu XB., Tsunekawa N., Kanai Y., Sone H., Tohyama C., and Kurohmaru M.	Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: A possible estrogenic effect of di(n-butyl) phthalate.	Reproduction	139	427-437	2010
Sone H, Okura M, Zaha H, Fujibuchi W, Taniguchi T, Akanuma H, Nagano R, Ohsako S, and Yonemoto J.	pCEC: a toxicogenomics database with a toxicoinformatics system for risk evaluation and toxicity prediction of environmental chemicals.	J Toxcol Sci	35	115-123	2010

別紙4

Ishihara K, Ohsako S, Tasaka K, Harayama H, Miyake M, Warita K, Tanida T, Mitsuhashi T, Nanmori T, Tabuchi Y, Yokoyama T, Kitagawa H, and Hoshi N.	When does the sex ratio of offspring of the paternal 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure decrease: In the spermatozoa stage or at fertilization?	Reprod Toxicol	29	68-73	2010
Ishimura R, Kawakami T, Ohsako S, and Tohyama C.	Dioxin-induced toxicity on vascular remodeling of the placenta.	Biochemical Pharmacol	77	660-669	2009
Kawakami T, Ito T, Ohsako S, Shiizakia K, Murakami Y, Hirowatarid K, Sato M, and Tohyama C.	Possible Involvement of arylhydrocarbon receptor variants in TCDD-induced thymic atrophy and XRE-dependent transcriptional activity in Wistar Hannover GALAS rats.	J Toxicol Sci,	34	209-220	2009
Wu Q., Suzuki J.S., Zaha H., Lin T-M., Peterson R.E., Tohyama C., and Ohsako S.	Differences in gene expression and benzo[a]pyrene-induced DNA adduct formation in the liver of three strains of female mice with identical AhRb2 genotype treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin and/or benzo[a]pyrene.	J Applied Toxicol	28	724-733	2008
Shiizaki K, Ohsako S, Kawanishi M, and Yagi T.	Omeprazole alleviates benzo[a]pyrene cytotoxicity by inhibition of CYP1A1 activity in human and mouse hepatoma cells.	Basic Clinical Pharmacol Toxicol	103	468-475	2008
Sakata Y., Yoshioka W., Tohyama C., and Ohsako S.	Internal genomic sequence of human CYP1A1 gene is involved in superinduction of dioxin-induced CYP1A1 transcription by	Biochem Biophys Res Comm	355	687-692	2007
1) Kondo Y, Nakajima Y, Komatsubara R, Kato M, Hirata N, Matuyama H, Kakami M, Tsuge I, Ohya Y, Urisu A.	Short term efficacy of tacrolimus ointment and impact on quality of life.	Pediatric International	51	385-9	2009

別紙4

Jonathan M Batchelor, Yukihiro Ohya	Use of DISCERN Instrument by patients and health professionals to assess information resources on treatment for asthma and atopic dermatitis.	Allergology International	58	141-145	2009
Miyake Y, Tanaka K, Sasaki S, Kiyohara C, Ohya Y, Fukushima W, Yokoyama T, Hirota Y.	Breast feeding and atopic eczema in Japanese infants: Osaka Maternal and Child Health Study.	Pediatr Allergy Immunolo	20	124-241	2009
Murakami K, Miyake Y, Sasaki S, Tanaka K, Ohya Y, Hirota Y.	Education, but not occupation or household income, is positively related to favorable dietary intake patterns in pregnant Japanese women: the Osaka Maternal and Child Health Stduy.	Nutrition Research	29	164-172	2009
Murakami K, Miyake Y, Sasaki S, Tanaka K, Ohya Y, Hirota Y.	Monetary diet cost is associated with not only favorable but also unfavorable aspects of diet in pregnant Japanese women: the Osaka Maternal and Child Health Study	Environmental Health Insight			2009
Kojima N, Ohya Y, Futamura M, Akashi M, Odajima H, Adachi U, Kobayashi F, Akasawa A.	Exercise-induced asthma is associated with impaired quality of life among children with asthma in Japan.	Allergology International	58	087-192	2009
7) Kubota Y, Yoneda K, Nakai K, Katsuura J, Moriue T, Matsuoka Y, Miyamoto I, Ohya Y.	Effct of sequential application of topical tacrolimus and topical corticosteroids in the treatment of pediatric atopic dermatitis: An open-label pilot study	J Am Acad Dermatol	60	212-217	2009
Kondo-Endo K, Ohashi Y, Nakagawa H, Katsunuma T, Ohya Y, Kamibeppu K, Masuko I.	Development and validation of a questionnaire measuring quality of life in primary caregivers of children with atopic dermatitis (QPCAD).	Br J Dermatol.			2009
赤澤晃 須田友子 明石昌幸 成田雅美 大矢幸弘 萬木暁美 渡辺博子 熊木みゆき 松本健治 斎藤博久	小児気管支喘息治療における吸入ステロイド薬中止後の臨床経過の前方視的研究	アレルギー	58	1407-1417	2009

別紙4

二村昌樹 伊藤浩明 尾辻健太 平山美香 林啓一 大矢幸弘 益子育代 :	乳幼児アトピー性皮膚炎患者に対する短期教育入院「スキンケアスクール」の効果	アレルギー	58	1610-1618	2009
福家辰樹 野村伊知郎 新井勝大 大矢幸弘:	症例報告＜小児科＞好酸球性食道炎の1小児例	アレルギーの臨床	29(13)	1182-1186	2009
大矢幸弘	アレルギー疾患治療のエンドポイント. 小児アトピー性皮膚炎	アレルギー	58	1375-1385	2009
Miyake Y, Tanaka K, Fujiwara H, Mitani Y, Ikemi H, Sasaki S, Ohya Y, Hirota Y.	Residential proximity to main roads during pregnancy and the risk of allergic disorders in Japanese infants: the Osaka Maternal and Child Health Study	Pediatr Allergy Immunol.	21(1 Pt 1)	22-28	2010
Saito K, Yokoyama T, Miyake Y, Sasaki S, Tanaka K, Ohya Y, Hirota Y.	Maternal meat and fat consumption during pregnancy and suspected atopic eczema in Japanese infants aged 3-4 months: the Osaka Maternal and Child Health Study.	Pediatr Allergy Immunol.	21(1 Pt 1)	38-46	2010
Kondo N, Nshimurta T, Nishima S, Morisawa A, Aihara Y, Akasaka T, Akasawa A, Adachi Y, Arakawa H, Ikarashi T, Ikebe Toshiichi, Inoue T, Iwata T, Urisu A, Ebisawa M, Ohya Y, Okada K, Odajima H, Katsunuma T, Kameda M, Kurihara K, Kohno Y, Sakamoto T, Sh	Japanese pediatric guideline for the treatment and management of bronchial asthma 2008	Pediatrics International	52	319-326	2010
Tanaka K, Miyake Y, Arakawa M, Sasaki S, Ohya Y	Household smoking and dental caries in schoolchildren: the Ryukyus Child Health Study.	BMC Public Health.	10	335	2010

別紙4

Fukuie T, Nomura I, Horimukai K, Manki A, Masuko I, Futamura M, Narita M, Ohzeki T, Matsumoto K, Saito H, Ohya Y.	Proactive treatment appears to decrease serum immunoglobulin-E levels in patients with severe atopic dermatitis	Br J Dermatol	163	1127-1129	2010
18) Okabe Y, Itazawa T, Adachi Y, Yoshida K, Ohya Y, Odajima H, Akasawa A, Miyawaki T.	Association of overweight with asthma symptoms in Japanese school children.	Pediatr Int.	in print		2010
Fujibuchi W, Kim H, Okada Y, Taniguchi T, Sone	H. High-performance gene expression module analysis tool and its application to chemical toxicity data.	Methods Mol Biol.	577	55-65.	
Alam MS, Ohsako S, Matsuwaki T, Zhu XB, Tsunekawa N, Kanai Y, Sone H, Tohyama C, Kurohmaru M.	Induction of spermatogenic cell apoptosis in prepubertal rat testes irrespective of testicular steroidogenesis: a possible estrogenic effect of di(n-butyl) phthalate.	Reproduction.	Feb;139(2)	427-37.	
Ohsako S, Fukuzawa N, Ishimura R, Kawakami T, Wu Q, Nagano R, Zaha H, Sone H, Yonemoto J, Tohyama C.	Comparative Contribution of the Aryl Hydrocarbon Receptor Gene to Perinatal Stage Development and Dioxin-Induced Toxicity Between the Urogenital Complex and Testis in the	Biol Reprod.			
Sone H, Okura M, Zaha H, Fujibuchi W, Taniguchi T, Akanuma H, Nagano R, Ohsako S, Yonemoto J.	Profiles of Chemical Effects on Cells (pCEC): a toxicogenomics database with a toxicoinformatics system for risk evaluation and toxicity prediction of environmental chemicals.	J Toxicol Sci.	35(1)	43	
森拓哉、吉永淳、河原純子、溝井美穂、安達修一	日本人小児の多環芳香族炭化水素類曝露評価	日本衛生学雑誌	64	817-823	
Y. Suzuki, M. Niwa, J. Yoshinaga, C. Watanabe, Y. Mizumoto, S. Serizawa, H. Shiraishi	Exposure assessment of phthalate esters in Japanese pregnant women by using urinary metabolite analysis	Environmental Health and Preventive Medicine	14	180-187	

別紙4

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
大迫誠一郎	エピジェネティクスと環境医学	松島綱治	分子予防環境医学	本の泉社	東京	2010	568-575
大迫誠一郎	プログラムされる"病"の新たな仮説ー環境化学物質による代謝系遺伝子の次世代エピゲノム変化.		科学	岩波書店	東京	2009	984-988
Sone H, Imanishi S, Akanuma H, Nagano R, Fukuda T, Ohsako S.	Gene expression signatures of environmental chemicals in cancer and in developmental disorders.		The roles of free radicals in biology and medicine	MEDIMOND SRL Publ.		2009	45-52
Sone H, Miyabara Y, Fukuda T, Okura M, Ohsako S, Yonemoto J.	Does maternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce glutathione S-transferase-positive hepatic foci in adult rats?	Morita M. ed.	Persistent organic Pollutants (POPS) Research in Asia			2008	257-261
Kojima Y, Mizuno K, Sasaki S, Hayashi Y, Kohri K	Gene therapy for male infertility; Potential and limitation	Lejeune T and Delvaux	Human spermatozoa: Maturation, Capacitation and Abnormalities.	Nova Science Publishers, Inc.,	New York	2010	221-242
Kojima Y, Casale P.	Pediatric robotic pyeloplasty for ureteropelvic junction obstruction	Ost MC	Laparoscopic reconstructive surgery in children and adults.	Springer	New York	2010	109-124
Kojima Y, Casale P.	Robotic Bladder Surgery in Children	Hemal AK and Menon M	Robotic Urologic Surgery	Springer	New York	in press	
Kojima Y, Sasaki S, Kubota Y, Hayashi S, Kohri K.	New Insights into Alpha1-Adrenoceptor Subtypes and Pharmacogenomics of Benign Prostatic Hyperplasia	Morrison EP	Horizons in Cancer Research	Nova Science Publishers	New York	2010	66-101

研究成果の刊行物・別刷り

CASE REPORT

Semen analysis and successful paternity by intracytoplasmic sperm injection in a man with steroid 5α-reductase-2 deficiency

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Objective: To report semen parameters and successful paternity by intracytoplasmic sperm injection (ICSI) in a male patient with molecularly confirmed steroid 5α-reductase-2 deficiency.
Design: Case report.
Setting: National research institute and an infertility clinic.
Patient(s): A 29-year-old Japanese man with 5α-reductase-2 deficiency who had failed to have a child despite an ordinary conjugal life for 2 years with his wife.
Intervention(s): Mutation analysis, semen analysis, and execution of ICSI.
Main Outcome Measure(s): Mutation detection, semen assessment, and production of a child.
Result(s): Mutation analysis revealed a homozygous p.R246Q missense mutation on exon 5 of *SRD5A2*. Semem analysis showed oligozoospermia (semen volume 0.3 mL, sperm count 15×10^6/mL, total sperm count 4.5×10^6, motile cells 17%, and normal morphologic sperm 8%). ICSI resulted in a production of a healthy male infant.
Conclusion(s): The results, in conjunction with those of previously reported patients who received semen analysis and/or achieved paternity, suggest that male patients with 5α-reductase-2 deficiency, especially those with hypomorphic mutations including p.R246Q, could retain some degree of spermatogenic function and achieve paternity with and without assisted reproductive technology. (Fertil Steril® 2010;94:2770.e7–e10. ©2010 by American Society for Reproductive Medicine.)

Key Words: Steroid 5α-reductase-2 deficiency, SRD5A2, hypomorphic mutation, semen analysis, intracytoplasmic sperm injection, paternity

Steroid 5α-reductase type 2 (5α-reductase-2) plays a crucial role in male sex differentiation by converting testosterone (T) into 5α-dihydrotestosterone (DHT) in the peripheral target tissues (1, 2). It is known that masculinization of wolffian ducts is primarily caused by T, whereas that of external genitalia and prostate is primarily caused by DHT (1). Therefore, 5α-reductase-2 deficiency, although it permits wolffian development, results in various degrees of 46,XY disorders of sex development (DSD) with undermasculinized or feminized external genitalia and hypoplastic prostate, depending on the residual enzyme activity (1–3).

Furthermore, semen quality also appears to be deteriorated in 5α-reductase-2 deficiency. Indeed, semen analysis in nine socially male patients derived from a single large Dominican pedigree revealed that sperm are low in number and reduced in motility, and that seminal plasma is characterized by markedly reduced volume, increased viscosity, and poor liquefaction (3, 4). To date, however, one of the nine Dominican male patients achieved paternity by intrauterine insemination (5), and two Swedish brothers with 5α-reductase-2 deficiency fathered children naturally (6, 7).

Here, we report semen data and successful paternity by intracytoplasmic sperm injection (ICSI) in a man with 5α-reductase-2 deficiency. The results provide further information about fertility in 5α-reductase-2 deficiency.

CASE REPORT

This Japanese male patient was born at term after an uncomplicated pregnancy and delivery. The parents were allegedly nonconsanguineous, and there was no individual with DSD in this pedigree. At birth, he was found to have micropenis, bilateral retractile testes, and penoscrotal hypospadias. Thus, he received testosterone enanthate injections (25 mg/dose, three times) for micropenis at 1 year of age, urethroplasty at the age of 6 years 5 months, and orchidopexy at age 9 years 11 months at a local hospital. Because endocrine studies at the time of orchidopexy showed an apparently normal serum T response to hCG stimulation as well as elevated gonadotropin values (actual data were not available), a provisional diagnosis of incomplete androgen insensitivity syndrome was made. Subsequently, he exhibited spontaneous pubertal development from his early teens. He worked as an office worker and married at 27 years of age. He always recognized himself as male, and never had a psychosocial problem.

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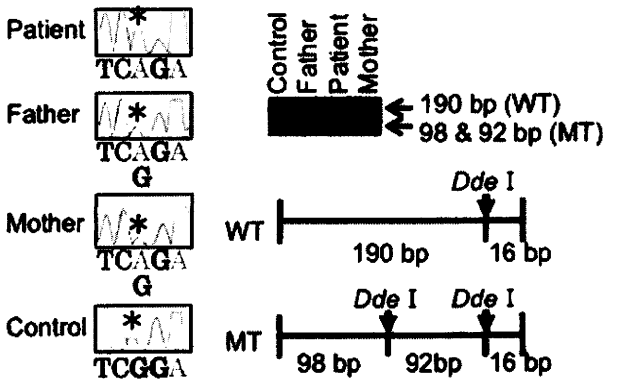
At 29 years of age, he came to us with his wife, because of infertility despite an ordinary conjugal life for 2 years. His penile length was 4.5 cm (age-matched normal: 9.0 ± 1.0 cm), pubic hair development at Tanner stage 4 (normal stage 4–5), and testis volume 10 mL bilaterally (normal 13–20 mL) (8–10). Wolffian structures (epididymides and vasa deferentia) were apparently normal, and there was no varicocele. Basal serum LH was 2.08 mIU/mL (normal 1.8–5.2 mIU/mL) (11), FSH 6.98 mIU/mL (normal 2.9–8.2 mIU/mL), T 6.56 ng/mL (normal 2.5–11.0 ng/mL), DHT 0.27 ng/mL (normal 0.23–0.85 ng/mL), and a T/DHT ratio 24.3 (normal 12 ± 3) (11, 12). His karyotype was 46,XY in all of the 50 lymphocytes examined. Therefore, he was diagnosed as having 5α-reductase-2 deficiency.

To confirm the diagnosis, mutation analysis was performed for *SRD5A2*, encoding steroid 5α-reductase-2. The present study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and was performed after obtaining written informed consent. In brief, leukocyte genomic DNA was amplified with the primers for all five exons and their flanking splice sites of *SRD5A2* by polymerase chain reaction (PCR), and the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA; the primer sequences are available on request). Consequently, a homozygous p.R246Q missense mutation was identified on exon 5 of the patient (Fig. 1). His parents were both heterozygous for this mutation. This mutation is predicted to create a *Dde*I restriction site, and this was confirmed by *Dde*I digestion of the PCR products harboring the mutation. The p.R246Q mutation was absent from 100 normal individuals. No sequence variation was identified in his wife. Furthermore, no discernible microdeletion was detected for the AZFa, AZFb, and AZFc regions on the Y chromosome (13) after analyzing multiple loci, including *RBMY* and *DAZ*.

Subsequently, semem analysis was performed after 7 days of abstinence. Semen volume was 0.3 mL (normal >2 mL), sperm count 15 × 10^6/mL (normal >20 × 10^6/mL), total sperm count 4.5×10^6 (normal >40 × 10^6), motile cells 17% (normal >50%), and normal morphologic sperm 8% (normal >30%; Table 1) (13). The couple selected ICSI after thorough consultation. A motile spermatozoa was microinjected into each of 20 mature (metaphase II) oocytes that were obtained after ovarian stimulation with FSH. Ten oocytes were fertilized and a single healthy-looking embryo was transfered to the uterus on day 3. This resulted in a successful production of a healthy 3.32 kg and 52.0 cm male infant at 41 weeks of gestation.

FIGURE 1

Mutational analysis of *SRD5A2*. The patient is homozygous for the p.R246Q missense mutation, and the parents are heterozygous for the mutation.



Matsubara. ICSI and steroid 5α-reductase-2 deficiency. Fertil Steril 2010.

DISCUSSION

This Japanese patient with 5α-reductase-2 deficiency had oligozoospermia and achieved paternity by ICSI. Because the p.R246Q mutation identified in this patient has been shown to be a hypomorphic mutation by functional studies (14, 15), it would explain why he had relatively mild clinical features, such as undermasculinized but obviously male genital development, unequivocally male gender role behavior, and a relatively low T/DHT ratio. It is likely that the residual enzymatic activity led to oligozoospermia rather than azoospermia, thereby permitting successful paternity by ICSI.

To date, twelve 46,XY patients with 5α-reductase-2 deficiency, including the present case, have received semen analysis and/or achieved paternity (Table 1) (4–7). They are invariably homozygotes for hypomorphic missense mutation (cases 1–10) or compound heterozygotes for hypomorphic missense mutations (cases 11 and 12), and there is no report documenting semem findings or paternity in patients with more severe mutations (e.g., patients with nonsense mutations on both alleles). It is assumed, therefore, that patients with residual activities have usually been reared as male, whereas those with more severe mutations have usually been raised as female.

Spermatogenic function was variable among the 12 patients, although external genitalia were invariably undermasculinized. Indeed, even in the same pedigree, cases 3 and 5–10 had severely impaired spermatogenesis, whereas case 2 had small semen volume and high sperm concentration and fathered three children by intrauterine insemination and case 4 showed oligozoospermia (4, 5). In addition, cases 11 and 12 retained fertility, although semen analysis was not performed (6, 7). Furthermore, such variability in spermatogenic function is apparently independent of the presence or absence of cryptorchidism (Table 1). Such variability in spermatogenic function, however, would not be unexpected, because, in contrast to external genital formation that occurs in the fetal life, spermatogenic function is influenced by multiple genetic and environmental factors for a long time (>20 y). Therefore, although residual enzymatic activities would have a certain effect on spermatogenic function, prediction of spermatogenic function appears to be difficult in patients with 5α-reductase-2 deficiency.

Nevertheless, it is noteworthy that four patients with 5α-reductase-2 deficiency, including the present case, achieved paternity with and without artificial reproductive technology (5–7). Recently, male gender assignment has been recommended for 46,XY patients with 5α-reductase-2 deficiency, primarily because they tend to show male gender role behavior (16, 17). The reports of successful paternity provide additional support for male gender assignment in 46,XY patients with 5α-reductase-2 deficiency, especially those with residual enzymatic activities.

When performing artificial reproductive techniques, including ISCI, in patients with genetic disorders for male infertility, thorough genetic counseling is required regarding the transmission risk of genetic abnormalities. Indeed, vertical transmission from father to son has been reported for a Yq deletion and an autosomal dominant mutation affecting spermatogenesis (18, 19).

TABLE 1

Summary of 46,XY patients with 5α-reductase-2 deficiency who received semem analysis and/or achieved paternity.

Patient			Genital findings		Endocrine data			Semen parameters					
Case no.	Age (y)	*SRD5A2* mutation	External genitalia	Testis vol., R, L (mL)	T (ng/mL)	DHT (ng/mL)	T/DHT ratio	Vol. (mL)	SC ($\times 10^6$/mL)	TSC ($\times 10^6$)	MC (%)	NM (%)	Paternity
1	29	p.R246Q (homo)	MP, HY, RT (B)	10, 10	6.56	27	24	0.3	15	4.5	17	8	ICSI
2	20	p.R246W (homo)	MP, HY	25, 20	11.00	0.29	38	0.15	321	48	56	71	
	34				6.81	0.12	57	0.5	165	83	33	ND	IUI
	36				6.69	0.10	67	0.5	65	33	66	ND	IUI
3	24	p.R246W (homo)	HY	14, 15	7.93	0.25	31	0.5	2	1	0	36	
4	21	p.R246W (homo)	HY	ND	11.06	0.18	61	0.4	12	4.8	10	59	
5	22	p.R246W (homo)	HY	18, 20	8.79	0.16	55	0.8	<1	<1	ND	ND	
6	24	p.R246W (homo)	HY	13, 13	8.18	0.17	48	0.1	<1	<1	0	<1	
7	22	p.R246W (homo)	HY	15, 12	10.4	0.51	20	1.0	0	0	0	0	
8	30	p.R246W (homo)	HY, CO (U)	ND	10.51	0.17	62	<0.05	<1	<1	0	4	
9	24	p.R246W (homo)	HY, CO (U)	15, 12	10.51	0.18	58	0.5	0	0	0	0	
10	20	p.R246W (homo)	HY, RT (B)	10, 8	8.61	0.23	37	0.2	0	0	0	0	
11	23[a]	p.H231R/p.G196S	MP, HY, CO	NA	8.93	0.19	47	ND	ND	ND	ND	ND	Natural
12	22[b]	p.H231R/p.G196S	MP, HY, CO	NA	2.7	ND	ND	ND	ND	ND	ND	ND	Natural
Normal value					2.5–11.0	0.23–0.85	12 ± 3	>2	>20	>40	>50	>30	
Reference					(11)	(11)	(12)	(13)	(13)	(13)	(13)	(13)	

Note: Case 1 is the present case, cases 2–10 are derived from a large pedigree (4, 5), and cases 11 and 12 are brothers (6, 7). All of the cases received surgical repair and/or testosterone treatment for undermasculinized external genitalia. B = bilateral; CO = cryptorchidism; DHT = dihydrotestosterone; HY = hypospadias; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; L = left; MC = motile cells; MP = micropenis; ND = not determined; NM = normal morphology; R = right; RT = retractile testis; SC = sperm count; T = testosterone; TSC = total sperm count; U = unilateral.

[a] Endocrine data were obtained at 16 years of age.

[b] Endocrine data were obtained at 14 years of age.

Matsubara. ICSI and steroid 5α-reductase-2 deficiency. Fertil Steril 2010.

For an autosomal recessive disease, if a spouse were heterozygous for the corresponding genes, half of children should have biallelic mutations for male infertility. Thus, we performed *SRD5A2* analysis in his wife to examine a possible transmission risk.

In summary, we observed successful paternity by ISCI in a male patient with molecularly confirmed 5α-reductase-2 deficiency and oligozoospermia. Further studies in various aspects, including spermatogenic function, will permit to set forth better management strategies in this condition.

REFERENCES

1. Wilson JD, Griffin JE, Russel DW. Steroid 5α-reductase 2 deficiency. Endocr Rev 1993;14:577–93.
2. Russel DW, Wilson JD. Steroid 5α-reductase two genes/two enzymes. Annu Rev Biochem 1994;63: 25–61.
3. Imperato-McGinley J, Gautier T, Zirinsky K, Hom T, Palomo O, Stein E, et al. Prostate visualization studies in male homozygous and heterozygous for 5α-reductase deficiency. J Clin Endocrinol Metab 1992;75:1022–6.
4. Cai LQ, Fratianni AM, Gautier T, Imperato-McGinley J. Dihydrotestosterone regulation of semen in male pseudohermaphrodites with 5α-reductase deficiency. J Clin Endocrinol Metab 1994;79:409–14.
5. Katz MD, Kligman I, Cai LQ, Zhu YS, Fratianni AM, Zervoudakis I, et al. Paternity by intrauterine insemination with sperm from a man with5α-reductase-2 deficiency. N Engl J Med 1997;336: 994–7.
6. Nordenskjold A, Ivarsson SA. Molecular characterization of 5α-reductase type 2 deficiency and fertility in a Swedish family. J Clin Endocrinol Metab 1998;83:3236–8.
7. Ivarsson SA. 5α-Reductase deficient men are fertile. Eur J Pediatr 1996;155:435.
8. Fujieda K, Matsuura N. Growth and maturation in the male genitalia from birth to adolescence II: change of penile length. Acta Paediatr Jpn 1987;29:220–3.
9. Matsuo N. Skeletal and sexual maturation in Japanese children. Clin Pediatr Endocrinol 1993;(2 Suppl):1–4.
10. Matsuo N, Anzo M, Sato S, Ogata T, Kamimaki T. Testicular volume in Japanese boys to the age of 15 years. Eur J Pediatr 2000;159:843–5.
11. Japan Public Health Association. Normal biochemical values in Japanese children. Tokyo: Sanko Press, 1996.
12. Imperato-McGinley J, Peterson RE, Gautier T, Cooper G, Danner R, Arthur A, et al. Hormonal evaluation of large kindred with complete androgen insensitivity: evidence for secondary 5α–reductase deficiency. J Clin Endocrinol Metab 1982;54:931–41.
13. Sigman M, Jarow JP. Male infertility. In: Wein AJ, Kavoussi LR, Novick AC, Partin AW, Peters CA, eds. Campbell-Walsh urology. 9th ed. Philadelphia: Saunders, 2006:609–53.
14. Wigley WC, Prihoda JS, Mowszowicz I, Mendonca BB, New MI, Wilson JD, et al. Natural mutagenesis study of the human steroid 5α-reductase 2 isozyme. Biochemistry 1994;33: 1265–70.
15. Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, et al. Molecular genetics of steroid 5α-reductase 2 deficiency. J Clin Invest 1992;90:799–809.
16. Wilson JD. The role of androgen in male gender role behavior. Endocr Rev 1999;20:726–37.
17. Lee PA, Houk CP, Ahmed SF, Hughes IA, International Consensus Conference on Intersex organized by the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. Consensus statement on management of intersex disorders. J Pediatr 2006;118:e488–500.
18. Hargreave TB. Genetics and male infertility. Curr Opin Obstet Gynecol 2000;207–19.
19. Sato N, Hasegawa T, Hori N, Fukami M, Yoshimura Y, Ogata T. Gonadotrophin therapy in Kallmann syndrome caused by heterozygous mutation of the gene for fibroblast growth factor receptor 1: report of three families: case report. Hum Reprod 2005;20:2173–8.

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Anorectal and urinary anomalies and aberrant retinoic acid metabolism in cytochrome P450 oxidoreductase deficiency

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ABSTRACT

Context: Cytochrome P450 oxidoreductase (POR) is an electron donor for all microsomal P450 enzymes including CYP26 involved in inactivation of all-trans retinoic acid (atRA). Although previous studies in *Por* knockout mice suggest that atRA accumulation is relevant to various posterior organ abnormalities, a systematic analysis has not been performed for anorectal and urinary anomalies in patients with POR deficiency (PORD).

Objective: To report the frequencies of anorectal and urinary anomalies and plasma atRA values in PORD patients.

Patients: We studied 37 Japanese patients with PORD, consisting of 15 homozygotes for R457H (group A), 15 compound heterozygotes for R457H and one apparently null mutation (group B), and seven patients with other combinations of mutations (group C). Since R457H is a severe hypomorphic mutation, the residual POR function is predicted to be higher in group A than in group B.

Results: Imperforate anus was observed in four patients (10.8%) and vesicoureteral reflux was found in three patients (8.1%), with no significant difference in the frequencies of such anomalies between groups A and B. In addition, a complex urogenital malformation including penile agenesis was identified in one patient. Plasma atRA values were above the reference range in nine of 12 patients examined, and were similar between groups A and B and between patients with and without anomalies.

Conclusions: The results imply that aberrant atRA metabolism due to CYP26 deficiency underlies various anorectal and urinary anomalies in patients with PORD. Clinical phenotypes may be primarily determined by maternal oral retinol intake during pregnancy, and plasma atRA values may be largely influenced by the amount of postnatal oral retinol intake in such patients.

Introduction

Cytochrome P450 oxidoreductase (POR) is an electron donor for all microsomal cytochrome P450 enzymes and several non-P450 microsomal enzymes [1]. Molecular abnormalities of *POR* lead to an autosomal recessive disorder characterized by skeletal dysplasia referred to as Antley–Bixler syndrome, adrenal dysfunction, 46,XX and 46,XY disorders of sex development (DSD), and maternal virilization during pregnancy [2,3]. Of these salient clinical features, skeletal dysplasia is primarily ascribed to impaired activities of POR-dependent cholesterolgenic enzymes CYP51A1 (lanosterol 14α-demethylase) and SQLE (squalene monooxygenase) in bone tissues, and the remaining features are primarily caused by defective activities of POR-dependent steroidogenic enzymes CYP17A1 (17α-hydroxylase and 17,20 lyase), CYP21A2 (21-hydroxylase), and CYP19A1 (aromatase) in adrenals, gonads, and placenta [3]. In addition, the backdoor pathway to dihydrotestosterone, which appears to take place in fetal adrenals, also plays a pivotal role in the development of 46,XX DSD [3,4]. Since all patients reported to date have at least one missense mutation with probable residual

Abbreviations: atRA, all-trans retinoic acid; IA, imperforate anus; POR, cytochrome P450 oxidoreductase; DSD, disorders of sex development; SQLE, squalene monooxigenase; RA, retinoic acid; RALDH, retinal dehydrogenase; PORD, POR deficiency; UTI, urinary tract infection; VUR, vesicoureteral reflux.

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functions, this suggests that complete loss of POR function is incompatible with life [3,4].

Retinoic acid (RA) is a signaling molecule involved in cell proliferation, differentiation, and apoptosis [5]. In human and murine fetuses, the most active form of RA, all-trans RA (atRA), is synthesized from maternally derived retinol by retinal dehydrogenase 2 (RALDH2/Raldh2) and retinol dehydrogenases, and converted into biologically inactive metabolites by CYP26/Cyp26 enzymes (primarily by CYP26A1/Cyp26a1 and partly by CYP26B1/Cyp26b1 and CYP26C1/Cyp26c1) [5–7]. The tissue concentration of atRA is tightly regulated by the balance between synthesis and inactivation, and both accumulation and deficiency of atRA lead to developmental defects [5,6]. Notably, murine embryos exposed to high-dose of atRA and those with a targeted deletion of *Cyp26a1* (*Cyp26a1*$^{-/-}$) manifest severe anomalies particularly in the hindgut, genital tubercle, and sacral/caudal vertebrae [8–10], indicating that posterior organs are highly susceptible to excessive atRA signaling.

Biological activities of CYP26 enzymes are supported by POR. Thus, it is predicted that POR deficiency (PORD) results in atRA accumulation because of impaired activities of CYP26 enzymes, leading to posterior organ anomalies. In support of this, *Por* knockout mice (*Por*$^{-/-}$), which are embryonic lethal, manifest severe posterior region anomalies comparable to those of *Cyp26a1*$^{-/-}$ mice [11]. Furthermore, previous studies have revealed that abnormal RA signaling takes place in the anorectal and urinary regions of *Por*$^{-/-}$ mouse embryos, and that phenotypes of these embryos are partially rescued by targeted deletion of *Raldh2* or by culturing the embryos in the serum-free medium (thus, retinol-free medium) [11]. These results imply that accumulation of atRA is involved in the development of posterior region abnormalities in *Por*$^{-/-}$ mice.

At present, however, a systematic phenotypic analysis has not been performed for posterior organ anomalies in PORD patients, although such anomalies have been described in a few of PORD patients [12,13]. Thus, we investigated anorectal and urinary anomalies and plasma atRA values in PORD patients.

Patients and methods

Patients

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development, and performed after obtaining written informed consent. We studied 37 Japanese patients with molecularly confirmed *POR* abnormalities (16 with 46,XY and 21 with 46,XX). Of the 37 patients, 35 have been reported previously [4]. The patients were classified into three groups on the basis of the mutation types: group A, homozygotes for the Japanese founder mutation R457H ($n = 15$); group B, compound heterozygotes for R457H and one apparently null mutation ($n = 15$); and group C, compound heterozygotes for other types of mutations ($n = 7$). Since R457H is a severe hypomorphic mutation with a low enzymatic activity [12], POR residual activity is predicted to be higher in group A than in group B, while it is unknown for group C.

Clinical assessment

Imperforate anus (IA) was evaluated by physical examination. Urinary anomalies such as vesicoureteral reflux (VUR) were assessed in patients with an episode(s) of urinary tract infection (UTI) by radiological studies including voiding cystourethrography and intravenous pyelography.

Measurement of plasma atRA values

Plasma atRA value was obtained by the previously described method [14]. In brief, a 2.5-ml aliquot of 0.1 ml phosphate buffer (pH 6.0) and 5 ml of ethyl ether were added to 1 ml of plasma. The mixture was vortexed and centrifuged at 3000 rpm for 15 min. A 4-ml aliquot of the upper layer was removed and evaporated to dryness, after which the residue was dissolved in methanol and analyzed by high-performance liquid chromatography using Nano-Space SI-2 pump (Shiseido, Tokyo) and a Capcell Pack-C18 UG120 column (Shiseido, Tokyo). The mobile phase consisted of 60% acetonitrile and 40% ammonium acetate buffer (v/v, 10%) at a flow rate of 1.5 ml/min. The samples were monitored using SPD-10A (Shimazu, Kyoto) with detection at 340 nm. The retention time of atRA was 7.5 min. Plasma atRA values of the patients were compared with reference values reported by Tang et al. [15].

Sequence analysis of HOX9–13 paralogs

To examine possible relevance of an additional gene mutation(s) in a patient with a complex phenotype including penile agenesis, we analyzed *HOX9–13* paralogs that are known to play a critical role in the urogenital and limb development [16]. In brief, leukocyte genomic DNA was PCR-amplified for all the coding exons and their flanking splice sites of *HOX9–13* paralogs, which are devoid of *HOXA12* and *HOXB10–12*, using the previously reported primers [17]. Subsequently, the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). When a substitution was identified, the corresponding sequence was studied in the parents and 100 control subjects.

Statistical analysis

The statistical significance of the mean was analyzed by the *t*-test, and that of the frequency was examined by the χ^2 test. $P < 0.05$ was considered significant.

Results

Anorectal and/or urinary anomalies

IA was observed in two patients of group A, one patient of group B, and one patient of group C, and VUR was found in one patient of each group (Table 1). There was no significant difference in the frequencies of IA and VUR between group A with two copies of R457H and group B with a single copy of R457H ($P = 0.60$ for IA and $P = 1.00$ for VUR). In addition, a complex urogenital malformation including penile agenesis, aplasia of the left kidney, duplication and malrotation of the right kidney, and VUR was identified in one patient of group B (case 10) (Fig. 1). There were no other clinically discernible anorectal or urinary anomalies.

Plasma atRA values

Plasma atRA was obtained in three patients of group A, seven patients of group B, and two patients of group C (Table 1). Plasma atRA values were above the reference range in nine patients and remained within the reference range in three patients. There was no significant difference in the atRA values between groups A and B (2.3 ± 0.3 ng/ml vs. 2.1 ± 0.4 ng/ml, $P = 0.45$) and between patients with and without anorectal and/or renal anomalies (1.8 ± 0.2 ng/ml vs. 2.5 ± 0.6 ng/ml, $P = 0.07$).

Sequence analysis of HOX9–13 paralogs in case 10

A novel heterozygous missense substitution (G3A) was identified for *HOXD13*. This substitution was found in a phenotypically normal father as well as in three of 100 control subjects. No other

Table 1
Anorectal/urinary anomalies and plasma atRA values in PORD patients.

Patients			*POR* mutations	Anorectal and urinary anomalies	Plasma atRA values (ng/ml)
Case	Karyotype	Age (y)	Amino acid changes		
Group A: Homozygotes for R457H					
1	46,XY	2.0	R457H/R457H	IA	N.M.
2	46,XX	0.4	R457H/R457H	Absent	N.M.
3	46,XX	14.1	R457H/R457H	Absent	**2.5**
4	46,XX	15.0	R457H/R457H	VUR (B)	**1.9**
5	46,XX	3.0	R457H/R457H	Absent	**2.5**
6	46,XX	0.1	R457H/R457H	IA	N.M.
Group B: Compound heterozygotes for R457H and an apparently null mutation					
7	46,XY	16.8	R457H/Q201X	Absent	**2.5**
8	46,XY	14.8	R457H/I444fsX449	Absent	**2.4**
9	46,XY	17.5	R457H/transcription failure	Absent	**1.9**
10	46,XY	2.1	R457H/R48fsX63	Complex[a]	1.5
11	46,XY	0.2	R457H/Q555fsX612	Absent	**2.6**
12	46,XX	9.0	R457H/IVS6+1G>A	Absent	1.6
13	46,XX	6.6	R457H/transcription failure	VUR (R)	**2.0**
14	46,XX	4.2	R457H/transcription failure	IA	N.M.
Group C: Other compound heterozygotes					
15	46,XY	0.4	R457H/A462-S463insIA	Absent	**3.7**
16	46,XY	18.0	R457H/L612-W620delinsR	VUR (R)	N.M.
17	46,XX	0.8	R457H/E580Q	Absent	N.M.
18	46,XX	0.7	R457H/348delV	IA	1.8

The values above the reference range are boldfaced.
Of 37 patients with PORD, 19 patients are not included in this table, because they had no anorectal or urinary anomalies and were not examined for plasma atRA values.
POR, cytochrome P450 oxidoreductase; atRA, all-trans retinoic acid; IA, imperforate anus; VUR, vesicoureteral reflux; R, right; B, bilateral; N.M., not measured.
Reference values: 1.0–1.8 ng/ml for atRA.
[a] Renal aplasia (L), renal duplication (R), renal malrotation, VUR (B), and penile agenesis.

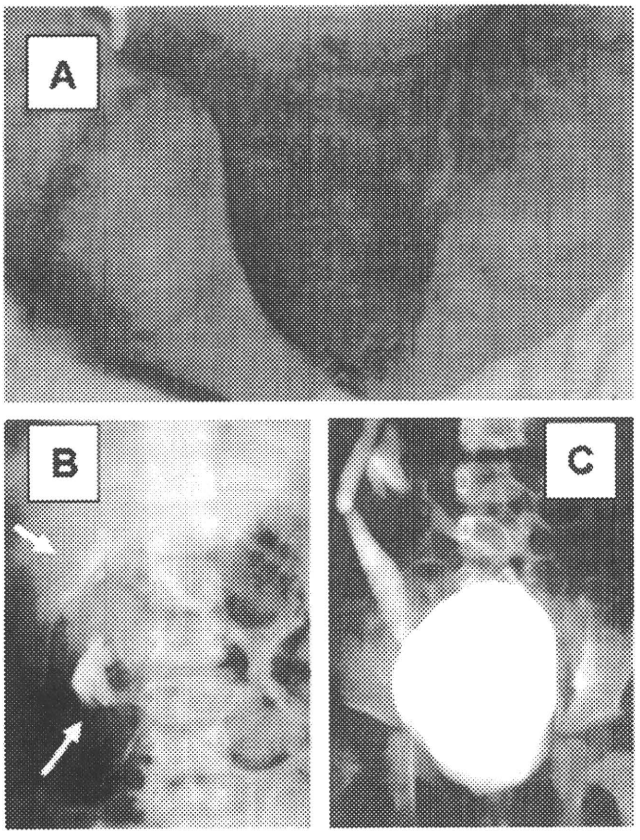

Fig. 1. Clinical phenotypes in case 10. (A) Penile agenesis. (B) Intravenous pyelography delineating aplasia of the left kidney and duplication and malrotation of the right kidney (arrows). (C) Voiding cystourethrography showing severe VUR.

sequence substitution was detected for the *HOX9–13* paralogs examined.

Discussion

We studied 37 patients with PORD and identified IA in four patients and VUR in three patients, as well as a complex urogenital malformation in a single patient. The frequencies of IA and VUR are obviously higher in PORD patients (10.8% for IA and 8.1% for VUR) than in the normal population (~0.0002% for IA and ~1.0% for VUR) [18,19]. In addition, the prevalence of VUR in the PORD patients may have been underestimated, because detailed urinary studies were carried out only in patients with an episode(s) of UTI. Thus, these findings imply that anorectal and urinary anomalies are characteristic features in PORD.

However, the frequencies of anorectal and urinary anomalies remained low, compared to those of salient clinical features such as skeletal dysplasia, adrenal dysfunction, DSD, and maternal virilization during pregnancy [4]. This suggests that a relatively low residual POR activity can permit normal anorectal and urinary development. Consistent with this, mice with a partial *Por* deletion that retains some residual activity lack anorectal and urinary anomalies, although they manifest severe limb defects [20]. Thus, PORD may function as a susceptibility or contributing factor, rather than a determinative factor, for the development of anorectal and urinary anomalies.

Plasma atRA values were elevated in most patients with PORD. This is compatible with reduced supporting activity of POR for CYP26 enzymes. Since plasma atRA is derived from specific atRA-producing tissues including liver and anorectal and urinary tissues [5], the atRA concentration may be drastically increased in such tissues. In this context, it is known that atRA functions as a paracrine or autocrine signaling factor [5] and regulates cellular apoptosis of the developing hindgut and the ureteric buds that are required for the normal anorectal and urinary development [21,22]. Thus, anorectal and urinary anomalies in PORD patients would primarily be ascribed to impaired CYP26 enzyme activities and resultant atRA accumulation in the anorectal and urinary regions. Indeed, liver-specific *Por* knockout mice have no discernible

abnormalities in the extra-hepatic organs including caudal regions, despite probably increased plasma atRA values because of impaired atRA inactivation in the liver [23].

Anorectal and urinary anomalies were identified in groups A–C with no difference in the prevalence between groups A and B, and plasma atRA values were similar between groups A and B and between patients with and without anorectal and urinary anomalies. In this regard, anorectal and urinary anomalies are generated in fetal life, and previous studies have indicated that phenotypic severity of *Por*$^{-/-}$, *Cyp26a1c1*$^{-/-}$, and *Cyp26a1b1c1*$^{-/-}$ mice embryos are obviously mitigated by a vitamin A-deficient diet and worsened by administration of a small dose of atRA during pregnancy [24,25]. Thus, while oral atRA intake would be neglectable in the daily human life, anorectal and urinary phenotypes in PORD patients may largely be influenced by the amount of maternal oral retinol intake during pregnancy. In addition, plasma atRA values in PORD patients would be influenced by the amount of postnatal oral retinol intake in such patients and would not reflect the amount of retinol transferred to such patients during pregnancy. These notions would explain the lack of correlation between the frequency of anomalies, postnatal plasma atRA values, and residual POR activities, although genetic factors such as polymorphisms of genes encoding atRA-synthesizing enzymes and/or RA binding protein would also be relevant to the variations in clinical phenotypes and plasma atRA values in PORD patients. Thus, it would be recommended to avoid retinol-rich foods during pregnancy of fetuses with a possibility of PORD.

A complex urogenital anomaly was identified in case 10. This phenotype may be explained as an extremely severe phenotype in PORD. In support of this, exposure to a large amount of retinol during pregnancy has caused renal and penile agenesis in a human patient [26]. Alternatively, there may be a hidden mutation(s) or susceptibility factor(s) in case 10, although we did not identify a definitive mutation in *HOX9–13* paralogs involved in the urogenital and limb development [16].

Several points should be made with respect to the present study. First, although a comprehensive skeletal survey was not performed in this study, impaired CYP26 activities in PORD may also lead to skeletal anomalies in the posterior region. Indeed, hemivertebrae and vertebral homeotic transformation have previously been identified in two PORD patients [12], and *Cyp26a1*$^{-/-}$ mice frequently exhibit vertebral anomalies such as spina bifida [9]. Second, impaired activities of POR-dependent enzymes other than CYP26 enzymes may also be relevant to anorectal and urinary anomalies. Indeed, reduced CYP51A1 and SQLE activities may affect the expression of several genes including *BMP4* and *FGF8* involved in posterior organ formation [27], because of intracellular cholesterol deficiency and resultant abnormal hedgehog signaling [28]. In support of this, Smith–Lemli–Opitz syndrome caused by mutations of *DHCR7* involved in cholesterolgenesis is often associated with Hirschsprung disease and renal malformations [29]. Lastly, while R457H is the most prevalent founder mutation in Japanese patients [4], A287P is the most common mutation in Caucasian patients [3,12]. In this context, supporting activities for POR-dependent steroidogenic enzymes are more severely compromised in R457H than in A287P [12] and, consistent with this, salient PORD phenotypes appear to be more severe in Japanese patients than in Caucasian patients [4,12]. Thus, while IA has also been identified in a Caucasian patient with A287P [12], the prevalence and the severity of anorectal and urinary anomalies remain to be clarified in different ethnic groups including Caucasian population.

In summary, the present study suggests that aberrant atRA metabolism due to CYP26 deficiency underlies anorectal and urinary anomalies in patients with PORD. Further studies will permit to define phenotypic spectrum in PORD and underlying factors for the development of clinical features.

Disclosure statement

The authors have nothing to declare.

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References

[1] M. Wang, D.L. Roberts, R. Paschke, T.M. Shea, B.S. Masters, J.J. Kim, Three-dimensional structure of NADPH–cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes, Proc. Natl. Acad. Sci. USA 94 (1997) 8411–8416.
[2] C.E. Flück, T. Tajima, A.V. Pandey, W. Arlt, K. Okuhara, C.F. Verge, E.W. Jabs, B.B. Mendonça, K. Fujieda, W.L. Miller, Mutant P450 oxidoreductase causes disordered steroidogenesis with and without Antley–Bixler syndrome, Nat. Genet. 36 (2004) 228–230.
[3] R.R. Scott, W.L. Miller, Genetic and clinical features of P450 oxidoreductase deficiency, Horm. Res. 69 (2008) 266–275.
[4] M. Fukami, G. Nishimura, K. Homma, T. Nagai, K. Hanaki, A. Uematsu, T. Ishii, C. Numakura, H. Sawada, M. Nakacho, T. Kowase, K. Motomura, H. Haruna, M. Nakamura, A. Ohishi, M. Adachi, T. Tajima, Y. Hasegawa, T. Hasegawa, R. Horikawa, K. Fujieda, T. Ogata, Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype–phenotype correlations in 35 Japanese patients, J. Clin. Endocrinol. Metab. 94 (2009) 1723–1731.
[5] G. Duester, Retinoic acid synthesis and signaling during early organogenesis, Cell 134 (2008) 921–931.
[6] S. Abu-Abed, P. Dollé, D. Metzger, B. Beckett, P. Chambon, M. Petkovich, The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures, Genes Dev. 15 (2001) 226–240.
[7] M. Taimi, C. Helvig, J. Wisniewski, H. Ramshaw, J. White, M. Amad, B. Korczak, M. Petkovich, A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid, J. Biol. Chem. 279 (2004) 77–85.
[8] Y. Sasaki, N. Iwai, T. Tsuda, O. Kimura, Sonic hedgehog and bone morphogenetic protein 4 expressions in the hindgut region of murine embryos with anorectal malformations, Pediatr. Surg. 39 (2004) 170–173.
[9] K. Niederreither, S. Abu-Abed, B. Schuhbaur, M. Petkovich, P. Chambon, P. Dollé, Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development, Nat. Genet. 31 (2002) 84–88.
[10] Y. Sakai, C. Meno, H. Fujii, J. Nishino, H. Shiratori, Y. Saijoh, J. Rossant, H. Hamada, The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo, Genes Dev. 15 (2001) 213–225.
[11] V. Ribes, D.M. Otto, L. Dickmann, K. Schmidt, B. Schuhbaur, C. Henderson, R. Blomhoff, C.R. Wolf, C. Tickle, P. Dollé, Rescue of cytochrome P450 oxidoreductase (Por) mouse mutants reveals functions in vasculogenesis, brain and limb patterning linked to retinoic acid homeostasis, Dev. Biol. 303 (2007) 66–81.
[12] N. Huang, A.V. Pandey, V. Agrawal, W. Reardon, P.D. Lapunzina, D. Mowat, E.W. Jabs, G. Van Vliet, J. Sack, C.E. Fluck, W.L. Miller, Diversity and function of mutations in P450 oxidoreductase in patients with Antley–Bixler syndrome and disordered steroidogenesis, Am. J. Hum. Genet. 76 (2005) 729–749.
[13] K. Homma, T. Hasegawa, T. Nagai, M. Adachi, R. Horikawa, I. Fujiwara, T. Tajima, R. Takeda, M. Fukami, T. Ogata, Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: implication for the backdoor pathway to dihydrotestosterone, J. Clin. Endocrinol. Metab. 91 (2006) 2643–2649.
[14] K. Takitani, H. Tamai, T. Morinobu, N. Kawamura, M. Miyake, T. Fujimoto, M. Mino, Pharmacokinetics of all-trans retinoic acid in pediatric patients with leukemia, Jpn. J. Cancer Res. 56 (1995) 400–405.
[15] G.W. Tang, R.M. Russell, 13-cis-Retinoic acid is an endogenous compound in human serum, J. Lipid Res. 30 (1990) 175–182.
[16] F.R. Goodman, P.J. Scambler, Human HOX gene mutations, Clin. Genet. 59 (2001) 1–11.
[17] K. Kosaki, R. Kosaki, T. Suzuki, H. Yoshihashi, T. Takahashi, K. Sasaki, M. Tomita, W. McGinnis, N. Matsuo, Complete mutation analysis panel of the 39 human HOX genes, Teratology 65 (2002) 50–62.

[18] R.E. Stevenson, Rectum and anus, in: R.E. Stevenson, J.G. Hall, R.M. Goodman (Eds.), Human Malformations and Related Anomalies, Oxford University Press, New York, 1993, pp. 493–499.
[19] J.S. Elder, Vesicoureteral reflux, in: R.M. Kliegman, R.E. Behrman, H.B. Jenson, B.F. Stanton (Eds.), Nelson Textbook of Pediatrics, Saunders, Elsevier, Inc., Philadelphia, 2007, pp. 2228–2234.
[20] A.L. Shen, K.A. O'Leary, C.B. Kasper, Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH–cytochrome P450 oxidoreductase, J. Biol. Chem. 277 (2002) 6536–6541.
[21] E. Batourina, S. Tsai, S. Lambert, P. Sprenkle, R. Viana, S. Dutta, T. Hensle, F. Wang, K. Niederreither, A.P. McMahon, T.J. Carroll, C.L. Mendelsohn, Apoptosis induced by vitamin A signaling is crucial for connecting the ureters to the bladder, Nat. Genet. 37 (2005) 1082–1089.
[22] R. Padmanabhan, Retinoic acid-induced caudal regression syndrome in the mouse fetus, Reprod. Toxicol. 12 (1998) 139–151.
[23] J. Gu, Y. Weng, Q.Y. Zhang, H. Cui, M. Behr, L. Wu, W. Yang, L. Zhang, X. Ding, Liver-specific deletion of the NADPH–cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase, J. Biol. Chem. 278 (2003) 5895–5901.
[24] M. Uehara, K. Yashiro, K. Takaoka, M. Yamamoto, H. Hamada, Removal of maternal retinoic acid by embryonic CYP26 is required for correct Nodal expression during early embryonic patterning, Genes Dev. 23 (2009) 1689–1698.
[25] D.M. Otto, C.J. Henderson, D. Carrie, M. Davey, T.E. Gundersen, R. Blomhoff, R.H. Adams, C. Tickle, C.R. Wolf, Identification of novel roles of the cytochrome p450 system in early embryogenesis: effects on vasculogenesis and retinoic Acid homeostasis, Mol. Cell. Biol. 23 (2003) 6103–6116.
[26] M.E. Martínez Tallo, E. Galán Gómez, J.L. Cordero Carrasco, E.H. Hidalgo Barquero, F.M. Campo Sampedro, J.J. Cardesa García, Penile agenesis and syndrome of multiple abnormalities associated with the ingestion of retinoic acid by the mother, An. Esp. Pediatr. 31 (1989) 399–400 (in Spanish).
[27] G. Yamada, K. Suzuki, R. Haraguchi, S. Miyagawa, Y. Satoh, M. Kamimura, N. Nakagata, H. Kataoka, A. Kuroiwa, Y. Chen, Molecular genetic cascades for external genitalia formation: an emerging organogenesis program, Dev. Dyn. 235 (2006) 1738–1752.
[28] M.K. Cooper, C.A. Wassif, P.A. Krakowiak, J. Taipale, R. Gong, R.I. Kelley, F.D. Porter, P.A. Beachy, A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis, Nat. Genet. 33 (2003) 508–513.
[29] G.E. Herman, Disorders of cholesterol biosynthesis: prototypic metabolic malformation syndromes, Hum. Mol. Genet. 12 (S1) (2003) R75–R88.

The IG-DMR and the *MEG3*-DMR at Human Chromosome 14q32.2: Hierarchical Interaction and Distinct Functional Properties as Imprinting Control Centers

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Abstract

Human chromosome 14q32.2 harbors the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes. Although previous studies in cases with microdeletions and epimutations affecting both DMRs and paternal/maternal uniparental disomy 14-like phenotypes argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, the precise role of the individual DMR remains to be clarified. We studied an infant with upd(14)pat body and placental phenotypes and a heterozygous microdeletion involving the IG-DMR alone (patient 1) and a neonate with upd(14)pat body, but no placental phenotype and a heterozygous microdeletion involving the *MEG3*-DMR alone (patient 2). The results generated from the analysis of these two patients imply that the IG-DMR and the *MEG3*-DMR function as imprinting control centers in the placenta and the body, respectively, with a hierarchical interaction for the methylation pattern in the body governed by the IG-DMR. To our knowledge, this is the first study demonstrating an essential long-range imprinting regulatory function for the secondary DMR.

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Introduction

Human chromosome 14q32.2 carries a cluster of protein-coding paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and non-coding maternally expressed genes (*MEGs*) such as *MEG3* (alias, *GTL2*), *RTL1as* (*RTL1* antisense), *MEG8*, *snoRNAs*, and *microRNAs* [1,2]. Consistent with this, paternal uniparental disomy 14 (upd(14)pat) results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax, abdominal wall defects, placentomegaly, and polyhydramnios [2,3], and maternal uniparental disomy 14 (upd(14)mat) leads to less-characteristic but clinically discernible features including growth failure [2,4].

The 14q32.2 imprinted region also harbors two differentially methylated regions (DMRs), i.e., the germline-derived primary *DLK1-MEG3* intergenic DMR (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR [1,2]. Both DMRs are hypermethylated after paternal transmission and hypomethylated after maternal transmission in the body, whereas in the placenta the IG-DMR alone remains as a DMR and the *MEG3*-DMR is rather hypomethylated [1,2]. Furthermore, previous studies in cases with upd(14)pat/mat-like phenotypes have revealed that epimutations (hypermethylation) and microdeletions affecting both DMRs of maternal origin cause paternalization of the 14q32.2 imprinted region, and that epimutations (hypomethylation) affecting both DMRs of paternal origin cause maternalization of the 14q32.2 imprinted region, while microdeletions involving the DMRs of paternal origin have no effect on the imprinting status [2,5–8]. These findings, together with the notion that parent-of-origin specific expression patterns of imprinted genes are primarily dependent on the methylation status of the DMRs [9], argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, with possible different effects between the body and the placenta.

However, the precise role of individual DMR remains to be clarified. Here, we report that the IG-DMR and the *MEG3*-DMR show a hierarchical interaction for the methylation pattern in the body, and function as imprinting control centers in the placenta and the body, respectively. To our knowledge, this is the first study demonstrating not only different roles between the primary and secondary DMRs at a single imprinted region, but also an essential regulatory function for the secondary DMR.

Author Summary

Genomic imprinting is a process causing genes to be expressed in a parent-of-origin specific manner—some imprinted genes are expressed from maternally inherited chromosomes and others from paternally inherited chromosomes. Imprinted genes are often located in clusters regulated by regions that are differentially methylated according to their parental origin. The human chromosome 14q32.2 imprinted region harbors the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes. Perturbed dosage of these imprinted genes, for example in patients with paternal and maternal uniparental disomy 14, causes distinct phenotypes. Here, through analysis of patients with microdeletions recapitulating some or all of the uniparental disomy 14 phenotypes, we show that the IG-DMR acts as an upstream regulator for the methylation pattern of the *MEG3*-DMR in the body but not in the placenta. Importantly, in the body, the *MEG3*-DMR functions as an imprinting control center. To our knowledge, this is the first study demonstrating an essential function for the secondary DMR in the regulation of multiple imprinted genes. Thus, the results provide a significant advance in the clarification of underlying epigenetic features that can act to regulate imprinting.

Results

Clinical reports

We studied an infant with upd(14)pat body and placental phenotypes (patient 1) and a neonate with upd(14)pat body, but no placental, phenotype (patient 2) (Figure 1). Detailed clinical features of patients 1 and 2 are shown in Table 1. In brief, patient 1 was delivered by a caesarean section at 33 weeks of gestation due to progressive polyhydramnios despite amnioreduction at 28 and 30 weeks of gestation, whereas patient 2 was born at 28 weeks of gestation by a vaginal delivery due to progressive labor without discernible polyhydramnios. Placentomegaly was observed in patient 1 but not in patient 2. Patients 1 and 2 were found to have characteristic face, small bell-shaped thorax with coat hanger appearance of the ribs, and omphalocele. Patient 1 received surgical treatment for omphalocele immediately after birth and mechanical ventilation for several months. At present, she is 5.5 months of age, and still requires intensive care including oxygen administration and tube feeding. Patient 2 died at four days of age due to massive intracranial hemorrhage, while receiving intensive care including mechanical ventilation. The mother of patient 1 had several non-specific clinical features such as short stature and obesity. The father of patient 1 and the parents of patient 2 were clinically normal.

Sample preparation

We isolated genomic DNA (gDNA) and transcripts (*mRNAs*, *snoRNAs*, and *microRNAs*) from fresh leukocytes of patients 1 and the parents of patients 1 and 2, from fresh skin fibroblasts of patient 2, and from formalin-fixed and paraffin-embedded placental samples of patient 1 and similarly treated pituitary and adrenal samples of patient 2 (although multiple body tissues were available in patient 2, useful gDNA and transcript samples were not obtained from other tissues probably due to drastic post-mortem degradation). We also made metaphase spreads from leukocytes and skin fibroblasts. For comparison, we obtained control samples from fresh normal adult leukocytes, neonatal skin fibroblasts, and placenta at 38 weeks of gestation, and from fresh leukocytes of upd(14)pat/mat patients and formalin-fixed and paraffin-embedded placenta of a upd(14)pat patient [2,3].

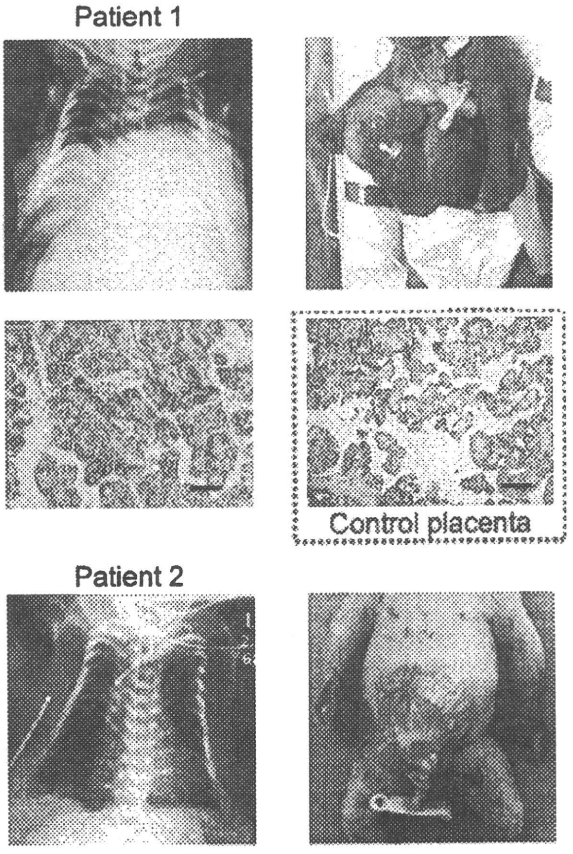

Figure 1. Clinical phenotypes of patients 1 and 2 at birth. Both patients have bell shaped thorax with coat hanger appearance of the ribs and omphalocele. In patient 1, histological examination of the placenta shows proliferation of dilated and congested chorionic villi, as has previously been observed in a case with upd(14)pat [2]. For comparison, the histological finding of a gestational age matched (33 weeks) control placenta is shown in a dashed square. The horizontal black bars indicate 100 μm.
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Structural analysis of the imprinted region

We first examined the structure of the 14q32.2 imprinted region (Figure 2). Upd(14) was excluded in patients 1 and 2 as well as in the mother of patient 1 by microsatellite analysis (Table S1), and FISH analysis for the two DMRs identified a familial heterozygous deletion encompassing the IG-DMR alone in patient 1 and her mother and a *de novo* heterozygous deletion encompassing the *MEG3*-DMR alone in patient 2 (Figure 2). The microdeletions were further localized by SNP genotyping for 70 loci (Table S1) and quantitative real-time PCR (q-PCR) analysis for four regions around the DMRs (Figure S1A), and serial direct sequencing for the long PCR products harboring the deletion junctions successfully identified the fusion points of the microdeletions in patient 1 and her mother and in patient 2 (Figure 2). According to the NT_026437 sequence data at the NCBI Database (Genome Build 36.3) (http://preview.ncbi.nlm.nih.gov/guide/), the deletion

Table 1. Clinical features in patients 1 and 2.

	Patient 1	Patient 2	Upd(14)pat (n = 20)[c]
Present age	5.5 months	Deceased at 4 days	0–9 years
Sex	Female	Female	Male:Female = 9:11
Karyotype	46,XX	46,XX	
Pregnancy and delivery			
Gestational age (weeks)	33	28	28–37
Delivery	Caesarean	Vaginal	Vaginal:Caesarean = 6:7
Polyhydramnios	Yes	No	20/20 (<28)[d]
Amnioreduction (weeks)	2× (28, 30)	No	6/6
Placentomegaly	Yes	No	10/10
Growth pattern			
Prenatal growth failure	No	No	1/13
Birth length (cm)	43 (WNR)[a]	34 (WNR)[a]	
Birth weight (kg)	2.84 (>90 centile)[a]	1.32 (WNR)[a]	
Postnatal growth failure	Yes	...	5/6
Present stature (cm)	56.3 (−3.0 SD)[b]	...	
Present weight (kg)	5.02 (−3.0 SD)[b]	...	
Characteristic face			
Frontal bossing	No	Yes	5/7
Hairy forehead	Yes	Yes	9/10
Blepharophimosis	Yes	No	14/15
Depressed nasal bridge	Yes	Yes	13/13
Anteverted nares	Yes	No	6/10
Small ears	Yes	Yes	11/12
Protruding philtrum	Yes	No	15/15
Puckered lips	No	No	3/10
Micrognathia	Yes	Yes	11/12
Thoracic abnormality			
Bell-shaped thorax	Yes	Yes	17/17
Mechanical ventilation	Yes	Yes	17/17
Abdominal wall defect			
Diastasis recti	...	...	15/17
Omphalocele	Yes	Yes	2/17[e]
Others			
Short webbed neck	Yes	Yes	14/14
Cardiac disease	No	Yes (PDA)	5/10
Inguinal hernia	No	No	2/6
Coxa valga	Yes	No	3/4
Joint contractures	Yes	No	8/10
Kyphoscoliosis	No	No	4/7
Extra features		Hydronephrosis (bilateral)	

WNR: within the normal range; SD: standard deviation; and PDA: patent ductus arteriosus.

a Assessed by the gestational age- and sex-matched Japanese reference data from the Ministry of Health, Labor, and Welfare (http://www.e-stat.go.jp/SG1/estat/GL02020101.do).

b Assessed by the age- and sex-matched Japanese reference data..

c In the column summarizing the clinical features of 20 patients with upd(14)pat, the denominators indicate the number of cases examined for the presence or absence of each feature, and the numerators represent the number of cases assessed to be positive for that feature; thus, the differences between the denominators and the numerators denote the number of cases evaluated to be negative for that feature (adopted from reference [2]).

d Polyhydramnios has been identified by 28 weeks of gestation.

e Omphalocele is present in two cases with upd(14)pat and in two cases with epimutations [2].

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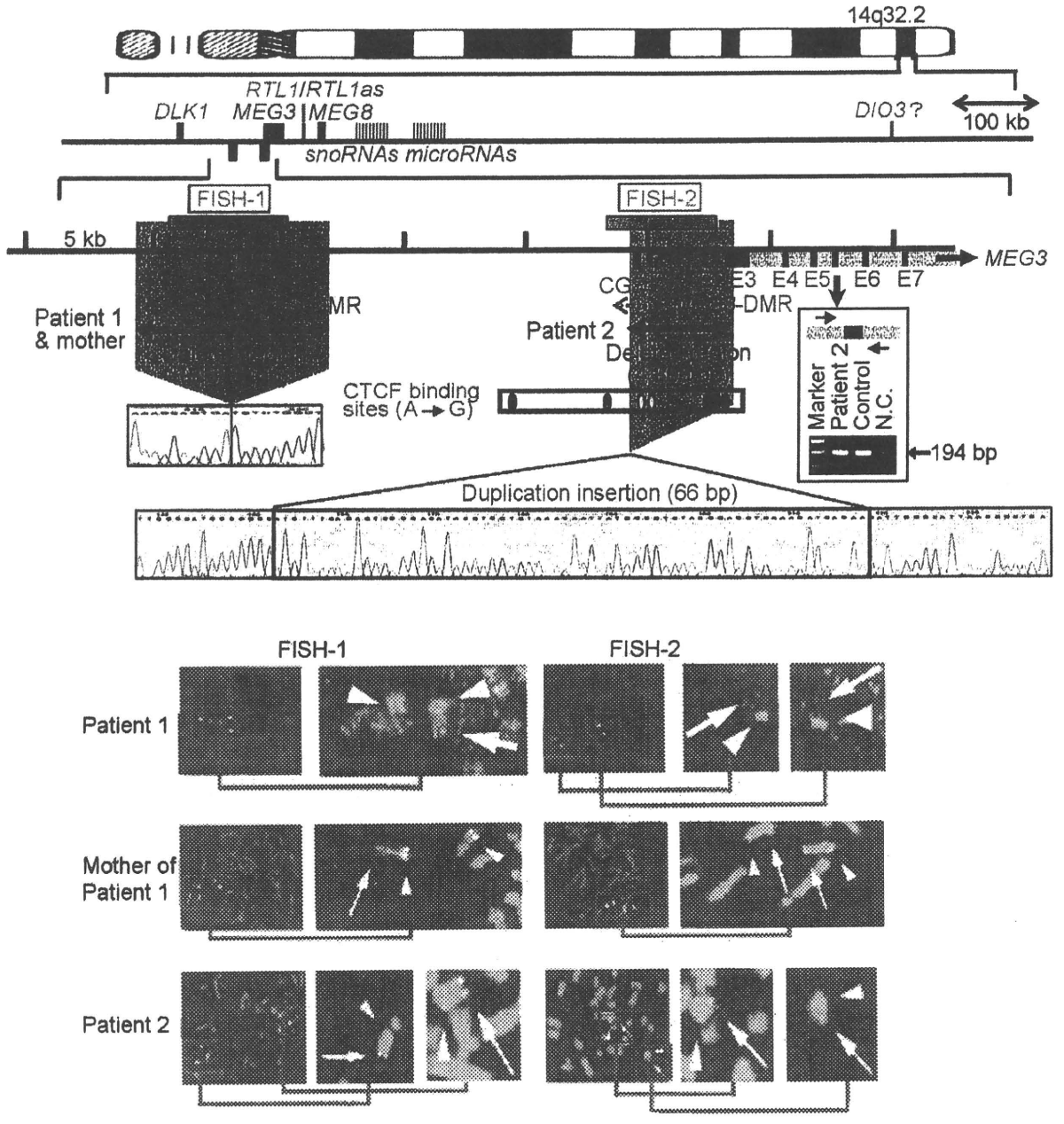

Figure 2. Physical map of the 14q32.2 imprinted region and the deleted segments in patient 1 and her mother and in patient 2 (shaded in gray). *PEGs* are shown in blue, *MEGs* in red, and the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) in green. It remains to be clarified whether *DIO3* is a *PEG*, although mouse *Dio3* is known to be preferentially but not exclusively expressed from a paternally derived chromosome [35]. For *MEG3*, the isoform 2 with nine exons (red bars) and eight introns (light red segment) is shown (Ensembl; http://www.ensembl.org/index.html). Electrochromatograms represent the fusion point in patient 1 and her mother, and the fusion point accompanied by insertion of a 66 bp segment (highlighted in blue) with a sequence identical to that within *MEG3* intron 5 (the blue bar) in patient 2. Since PCR amplification with primers flanking the 66 bp segment at *MEG3* intron 5 has produced a 194 bp single band in patient 2 as well as in a control subject (shown in the box), this indicates that the 66 bp segment at the fusion point is caused by a duplicated insertion rather than by a transfer from intron 5 to the fusion point (if the 66 bp is transferred from the original position, a 128 bp band as well as a 194 bp band should be present in patient 2) (the marker size: 100, 200, and 300 bp). In the FISH images, the red signals (arrows) have been identified by the FISH-1 probe and the FISH-2 probe, and the light green signals (arrowheads) by the RP11-566I2 probe for 14q12 used as an internal control. The faint signal detected by the FISH-2 probe in patient 2 is consistent with the preservation of a ~1.2 kb region identified by the centromeric portion of the FISH-2 probe.
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