析の結果までもが不可欠となる。そこで、このようなセンシティブ情報の取扱いについて、倫理的な側面から検討することが必要となった。

本分担研究では、まず文部科学省・厚生労働省・経済産業省による「ヒトゲノム・遺伝子解析研究に関する倫理指針」（平成16年12月28日全部改正、平成17年6月29日一部改正）において、臨床的遺伝子診断と診断研究（遺伝子解析研究）の区別をどのように整理しているかを参照し、当該コンサルテーション・システムの運用に際して、今後どのような整理（もしくはルール作り）が必要であるかを検討した。

また、他施設への遺伝学的相談を行うという状況において、研究領域での相談行為と臨床診断領域での相談行為の手続きの相違についても具体的に検討した。

（倫理面への配慮）

本分担研究は、理論研究であり、人ならびに人由来資料を用いることのない研究であることから、倫理的問題はないと考える。

C．研究結果

1）文部科学省・厚生労働省・経済産業省による「ヒトゲノム・遺伝子解析研究に関する倫理指針」（平成16年12月28日全部改正、平成17年6月29日一部改正）における臨床的遺伝子診断と診断研究（遺伝子解析研究）の線引き

文部科学省・厚生労働省・経済産業省による「ヒトゲノム・遺伝子解析研究に関する倫理指針」（平成13年3月29日策定、平成16年12月28日全部改正、平成17年6月29日一部改正）における臨床的遺伝子診断と診断研究（遺伝子解析研究）の線引きは、第1　2 本指針の適用範囲 (1)によって読み取ることができる。（表1）すなわち、

「…なお、診療において実施され、解析結果が提供者及びその血縁者の診療に直接生かされることが医学的に確立されている臨床検査及びそれに準ずるヒトゲノム・遺伝子解析は、医療に関する事項として、今後、慎重に検討されるべき課題であり、本指針の対象としない。」とあり、臨床的に診断・治療に生かされることが医学的に確立している場合は当該研究の倫理指針の対象としないとされている。

では、「医学的に確立している場合」とはどのような要件を満たしている場合を指すのであろうか。

これに対する規制当局の見解は、ヒトゲノム遺伝子解析研究に関する倫理指針Q&A（平成17年3月18日）の中に記載されている。すなわち、「『医学的に確立』されているとは、例えば医療保険適用となっている、学会においてガイドラインで示されているなど、一般的に当該検査の妥当性が認められている場合であり、探求的な位置づけで行われるものはこれには該当しません。このような当該検査の社会的評価及び位置づけを踏まえ、総合的に判断するものと考えます。」とし、具体的な判断基準の明記は避け、学会等の専門家集団によるガイドライン等に判断を委ねた形になっている。

現在、遺伝子診断に関係する諸学会（遺伝医学関連学会）として、日本遺伝カウンセリング学会、日本遺伝子診療学会、日本産科婦人科学会、日本小児遺伝学会、日本人

類遺伝学会、日本先天異常学会、日本先天代謝異常学会、日本マススクリーニング学会、日本臨床検査医学会、日本家族性腫瘍学会の10学会がある。これらの遺伝医学関連学会は、平成15年8月に「遺伝学的検査に関するガイドライン」と策定してはいるが、これに先立ち策定されていた「ヒトゲノム・遺伝子解析研究に関する倫理指針」（平成13年3月29日策定）に中にある

「（略）これらのヒトゲノム・遺伝子解析についても、診療を行う医師の責任において、関係学会等において作成される指針等を参考に、本指針の趣旨を踏まえた適切な対応が望まれる。」に対する前向きな対応はなされていない。というよりも、当該遺伝医学関連学会では反対に「遺伝学的検査に関するガイドライン」の「おわりに」の中で、「…今後は，日本遺伝子診療学会が要望したように[註]，また他国でも指摘されているように，遺伝学的検査そのものの公的機関による評価体制，監視体制を整える必要がある．とくに，遺伝学的検査の分析的妥当性，臨床的妥当性，臨床的有用性が十分なレベルにあることを確認するための公的審査機関の設置，及び常に新しい情報の提供と診断精度の向上を図るため，検査後の追跡調査をふくめ，公的機関による精度管理の実施などが必要である．（略）」

さらにガイドライン末尾の「提言」の中で「(1) 遺伝学的検査の分析的妥当性，臨床的妥当性，臨床的有用性が十分なレベルにあることを確認するため，公的審査機関の設置が必要である．(2) 遺伝学的検査を担当する施設は，常に新しい情報を得て，診断精度の向上を図るため，検査後の追跡調査をふくめ，公的機関などによる一定の（精度）管理の下に置かれるべきである．（以下、省略）」として、国に公的な審査・判断機関の設置を強く求めている。

これに対し、国としての具体的対応は特に見られず、平成16年12月の指針全部改正ならびに平成17年6月の一部改正の際にも、引き続き関係学会等のガイドライン作成に期待しているようである。

2）臨床的遺伝子診断の場合と診断研究（遺伝子解析研究）の場合の個人情報保護等の考え方の相違

臨床的遺伝子診断と診断研究（遺伝子解析研究）の根本的な違いは、その第一目的が「被検者の診断のためである」のか「研究のためである」のか、という点であり、また結果が「被検者及びその血縁者の診療に直接生かされることが医学的に確立されている」か否かである。

もし臨床的な診断であるならば、被検者への結果の開示（臨床へのフィードバック）が前提となるので、患者情報の匿名化の実施については、慎重に検討しなければならない。すなわち、匿名化することによる情報の取り違いのリスクと匿名化しないことによる個人情報漏洩のリスク等のバランスを考えなければならないのである。

当該研究班にて構築を進めている診断コンサルテーション・システムでは、昨年度の報告書にまとめた通り、極めて高いセキュリティレベルを確保していることから、相談時の個人情報漏洩のリスクは限りなく低いと推察されるが、遺伝子解析検査を外部機関に依頼する場合の個人情報漏洩のリスクも実務的には考えなければならない。

しかしながら、いずれにしても臨床的な診断である限りにおいては、正確な診断を優先するとして匿名化せずに検査を進めることは認められていると考えられる。

問題は、臨床的遺伝子診断という評価が十分に定まっていない遺伝子解析を必要とする診断の場合である。この場合、研究として症例集積をすることを目的とはしているが、臨床診断においても遺伝子解析結果がある程度参考になる検査もあると推察される。しかし、これらは個々の遺伝子検査法の精度に依存することになる。

このように臨床的に（医学的に）妥当性や有用性が認められるとする判断基準は、前述の通り現段階では明示されたものがない。例えば、1) 検査の精度が高く安定している、2) 検査結果に基づく早期予防もしくは治療が可能、等の項目についての具体的な整理は現段階でも可能と考える。そして、臨床的な検査か否かをその時その時にあった基準で線引きすることにより、臨床的な検査についてはより適正かつ公正に実施できると考える。また、研究的な色彩が強いと判断された検査については、ゲノム指針を遵守することにより、個人情報保護も含め、より適正な実施が確保できるだろう。

D．考察

残念なことに、本邦では、臨床診断と診断研究の整理がなされていないのが現状である。具体的には、平成13年に規制当局は学会に判断基準の作成を求め、平成14年に日本遺伝子診療学会が緊急アピールとして規制当局による判断基準の作成を求め、さらに平成15年に遺伝医学関連学会（10学会）がそのガイドラインの中で規制当局に再度求め、平成16年のゲノム指針全部改正の際に、さらに規制当局が学会に求めた…が、未だいずれにも具体的な対応はないという状況にあるようである。

このように規制当局ならびに専門家集団（学会）が互いに臨床的遺伝子診断と診断研究（遺伝子解析研究）の線引きの必要性を認めながらも、相互にその判断基準の作成の具体的な役割と責任を要請しあっている状況は、現在も続いていると推察される。

E．結論

まずは、早急に「医学的に確立されている臨床検査」についての具体的な整理を行なうことが必要であろう。しかしながら、これらの基準は、医科学技術の急速な進歩に伴い日々見直しがなされなければならない。そのためには、恒常的に機能できる公的な審査・判断機関の設置の検討も必要であろう。また、判断基準の整理には専門家集団の知見が不可欠である。したがって、国と専門家集団の協力により、一刻も早い判断基準の整備と検査の精度管理等が求められていると考えられる。

いずれにしろ、これらの臨床行為か否かの判断基準がないことは、診断精度の管理の問題等、被検者である患者の不利益を生み出す。特に、遺伝子解析を伴う診断行為の検査費用ならびに診療報酬の適正な設定の問題にも影響を及ぼしていると考えられることから、適正な価格で遺伝子診断を受ける患者の権利を侵害している可能性も否めない。したがって、早急に検討が必要な

課題と考える。

註

「遺伝子検査の妥当性と有用性に関する評価機構の早期設置を要望する緊急アピール」．日本遺伝子診療学会．2002

F．研究発表

なし。

表1　ゲノム指針の適用範囲（遺伝子解析研究として規制を受ける範囲）

第1　2 本指針の適用範囲 (1)

本指針は、ヒトゲノム・遺伝子解析研究を対象とし、その研究に携わる研究者等に遵守を求めるものである。適正な研究の実施のためには、研究者等一人ひとりの努力が重要であるほか、研究を行う機関においても個人情報の保護や倫理面での対応を適切に行うために必要な組織体制や環境の整備を図ることが重要である。

なお、診療において実施され、解析結果が提供者及びその血縁者の診療に直接生かされることが医学的に確立されている臨床検査及びそれに準ずるヒトゲノム・遺伝子解析は、医療に関する事項として、今後、慎重に検討されるべき課題であり、本指針の対象としない。

ただし、これらのヒトゲノム・遺伝子解析についても、診療を行う医師の責任において、個人情報の保護に関する法律に基づく医療・介護関係事業者における個人情報の適切な取扱いのための指針に従うとともに、関係学会等において作成される指針等を参考に、本指針の趣旨を踏まえた適切な対応が望まれる。

（下線は筆者による）

表2. ヒトゲノム遺伝子解析研究に関する倫理指針Q&A（平成17年3月18日）より

Q: 「医学的に確立されている臨床検査」は本指針の対象とならないとされていますが、「医学的に確立」とは、どのような基準で判断すればよいのでしょうか。

A: 「医学的に確立」されているとは、例えば医療保険適用となっている、学会においてガイドラインで示されているなど、一般的に当該検査の妥当性が認められている場合であり、探求的な位置づけで行われるものはこれには該当しません。このような当該検査の社会的評価及び位置づけを踏まえ、総合的に判断するものと考えます。

表3 ゲノム指針における「診療」の線引き

旧ゲノム指針（平成13年3月策定）	現行ゲノム指針（平成16年12月全部改正）
なお、診療において実施され、解析結果が提供者及びその血縁者の診療に直接生かされることが医学的に確立されている臨床検査及びそれに準ずるヒトゲノム・遺伝子解析は、医療に関する事項として、今後、慎重に検討すべき課題であり、本指針の対象としない。ただし、これらのヒトゲノム・遺伝子解析についても、診療を行う医師の責任において、関係学会等において作成される指針等を参考に、本指針の趣旨を踏まえた適切な対応が望まれる。	なお、診療において実施され、解析結果が提供者及びその血縁者の診療に直接生かされることが医学的に確立されている臨床検査及びそれに準ずるヒトゲノム・遺伝子解析は、医療に関する事項として、今後、慎重に検討されるべき課題であり、本指針の対象としない。ただし、これらのヒトゲノム・遺伝子解析についても、診療を行う医師の責任において、個人情報の保護に関する法律に基づく医療・介護関係事業者における個人情報の適切な取扱いのための指針に従うとともに、関係学会等において作成される指針等を参考に、本指針の趣旨を踏まえた適切な対応が望まれる。

（下線は筆者による）

研究成果の刊行物・別刷り

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CASE REPORT

Hypogonadotropic hypogonadism in an adult female with a heterozygous hypomorphic mutation of *SOX2*

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Abstract

Objective: Heterozygous *SOX2* mutations have recently been reported to cause isolated hypogonadotropic hypogonadism (HH), in addition to ocular and brain abnormalities. Here, we report a further case with a heterozygous hypomorphic *SOX2* mutation and isolated HH.

Patient: The patient was a 28-year-old Japanese female with congenital right anophthalmia and poor pubertal development, who was found to have HH by a gonadotropin-releasing hormone test (peak serum LH, 2.3 mIU/ml; peak serum FSH, 2.9 mIU/ml). Other pituitary hormones were normal.

Methods: We performed mutation analysis of *SOX2* and functional studies of mutant SOX2 protein using the core enhancer sequence of the chicken δ-1-crystallin gene (*DC5*) and that of the mouse nestin gene (*Nes30*).

Results: A heterozygous missense mutation (224T>A, Leu75Gln) was identified in the DNA-binding domain. The mutant SOX2 protein had a severely reduced (approximately 10%) DNA-binding affinity and a markedly diminished (20–30%) transactivation potential with no dominant negative effect.

Conclusions: The results provide further support for the positive role of *SOX2* in the regulation of gonadotropin production.

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Introduction

Hypogonadotropic hypogonadism (HH) is a genetically heterogeneous condition defined by the deficiency of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion (1). It can occur as an isolated form or in association with other pituitary hormone deficiency. To date, several causative genes have been identified in isolated HH, including *GNRHR*, *GPR54*, *DAX1*, *SF-1*, *KAL1*, and *FGFR1* (2, 3). Of these, mutations of *GNRHR* and *GPR54* are free from clinical features other than isolated HH, whereas those of the remaining genes are usually associated with characteristic clinical phenotypes in addition to isolated HH (2, 3). However, mutations of such genes account for a relatively small fraction of patients with isolated HH (1), and underlying genetic factors remain to be elucidated in many patients with this condition.

SRY-related high mobility group (HMG) box gene 2 (*SOX2*) is a single exon gene encoding a member of SOX transcription factor family involved in the regulation of embryonic development and in the determination of cell fate (4–6). SOX proteins bind specific DNA sequences through their HMG domain, and regulate specific downstream target genes by interacting with a variety of partner proteins (6). *SOX2* is expressed in multiple developing tissues including the eyes and the central nervous system (CNS) (5, 7, 8), and paired box gene 6 and brain 2 act as partner proteins of SOX2 in transcriptional regulation during the lens and the CNS development respectively (8, 9). Consistent with this, heterozygous loss-of-function mutations of *SOX2* are known to cause ocular and CNS abnormalities (10, 11).

Recently, Kelberman *et al.* (12) have reported that heterozygous *SOX2* mutations cause anterior pituitary hypoplasia and apparently isolated HH. Six patients with functionally impaired *de novo SOX2* mutations invariably had HH, and exhibited clinical features consistent with HH such as micropenis and/or cryptorchidism in affected males and delayed or lack of pubertal development in affected females. Consistent with this, micropenis and/or cryptorchidism have previously been described in some male patients with *SOX2* mutations (10, 11).

However, there has been no other report documenting the association between a *SOX2* mutation and HH. Here, we report a further case with the association.

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Subject and methods

Case report

This Japanese female patient was born at 36 weeks of gestation, with a birth length of 43.0 cm (−2.8 S.D.) and birth weight of 2.2 kg (−2.2 S.D.). She had congenital right anophthalmia, and received cosmetic repair with an artificial eye at 3 years of age. The non-consanguineous parents and the younger brother were clinically normal.

At 28 years of age she was referred to us, because of primary amenorrhea. Her height was 152.3 cm (−1.1 S.D.) and her weight 45.5 kg (−1.0 S.D.). Physical examination showed poor pubertal development (breast, Tanner stage 1; pubic hair, Tanner stage 2) with no virilization. Endocrine studies indicated isolated HH (Table 1), and her bone age was assessed as 15 years by the TW-2 method standardized for Japanese (15). Chromosome analysis revealed a 46,XX karyotype in all the 50 lymphocytes examined. Brain-computed tomography delineated right anophthalmia and apparently normal left eye and pituitary gland. The visual acuity of her left eye was 0.4 with a naked eye and 1.2 with a glass. Abdominal ultrasound studies indicated hypoplastic uterus and failed to detect ovaries. After consultation, she received oral hormone replacement therapy rather than gonadotropin therapy. She worked as a cook and had apparently normal mental development, although the measurement of intelligence quotient was refused.

Mutation analysis

This study was approved by the Institutional Review Board Committees at National Center for Child Health and Development and Osaka University. After obtaining written informed consent, leukocyte genomic DNA of this patient was amplified by PCR for the single coding exon and flanking UTRs of *SOX2*, using the primers shown in Table 2. Subsequently, the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA, USA). To confirm a heterozygous mutation, the corresponding PCR product was subcloned with TOPO TA Cloning Kit (Invitrogen), and normal and mutant alleles were sequenced separately. For controls, DNA samples of 100 normal individuals were utilized with permission.

DNA-binding assay

DNA-binding affinity of SOX2 HMG domain was examined for wildtype (WT) and mutant (MT) proteins using the core enhancer sequence at intron 3 of the chicken δ-1-crystallin gene (designated as DC5) harboring a SOX2 protein binding site (9). The detailed methods have been reported previously (9). In short, WT and MT SOX2 proteins (amino acids 2–184) tagged with 6xHis at the C-terminus were expressed in *Escherichia coli* as glutathione S-transferase (GST)-fusion proteins using expression vector pGEX-6P-1 (Amersham Pharmacia), and the GST tag was removed with PreScission protease. Varying amounts of the recombinant SOX2 proteins were incubated with the ^{32}P end-labeled DC5 and subjected to gel electrophoresis.

Transactivation analysis

Transactivation potential was examined with the Dual Luciferase Reporter Assay system (Promega), using DC5 containing the binding sites for SOX2 and chicken Pax6 proteins (9) and the core enhancer sequence at intron 2 of the mouse nestin gene (designated as Nes30) harboring the binding sites for SOX2 and mouse Brn2 proteins (8). The detailed methods were as described

Table 1 Summary of blood endocrine data.

	Patient data		Reference data	
	Baseline	Stimulated	Baseline	Stimulated
Luteinizing hormone (LH; mIU/ml)	<0.1	2.3[a]	1.8–7.6	8.5–15.5
Follicle-stimulating hormone (FSH; mIU/ml)	0.4	2.9[a]	5.2–14.4	8.3–20.0
Growth hormone (GH; ng/ml)	1.9	23.6[b]	0.6–3.5	>15.0
Adrenocorticotropic hormone (ACTH; pg/ml)	39	61[c]	9–52	>50
Thyroid-stimulating hormone (μU/ml)	0.6	14.8[d]	0.3–3.5	>10
Prolactin (ng/ml)	12.3	44.5[d]	1.5–15	5–70
Estradiol (pg/ml)	10	30[e]	20–120	300–1300
Testosterone (ng/ml)	<0.1	<0.1[f]	0.1–0.6	No female data
Insulin-like growth factor-I (ng/ml)	220	–	202–403	–
Cortisol (μg/dl)	18	22[g]	4–18	>20
Free thyroxine (pg/ml)	3.12	–	2.47–4.34	–
Free tri-iodothyronine (ng/dl)	1.56	–	0.97–1.79	–

Stimulated values represent (1) peak values during [a]a gonadotropin-releasing hormone test (100 μg bolus i.v), [b]a growth hormone-releasing hormone test (100 μg bolus i.v.), [c]a corticotropin-releasing hormone test (100 μg bolus i.v.), and [d]a thyrotropin-releasing hormone test (500 μg bolus i.v.) (blood sampling at 0, 30, 60, 90, and 120 min); (2) [e]the values after human menopausal gonadotropin stimulation (150 IU i.m. for 3 consecutive days) and [f]human chorionic gonadotropin stimulation (5000 IU i.m. for 3 consecutive days) (blood sampling on day 4); and (3) [g]the value after ACTH stimulation (250 μg i.v.) (blood sampling at 30 min). Reference data indicate the normal ranges in adult Japanese females (13, 14); those for LH and FSH indicate values at a follicular phase.

Table 2 The primer sequences and the PCR conditions utilized in the present study.

Primer	Forward primer; reverse primer	Location (bp)[a]	AT (C); PS (bp)
SOX2-1	CCGCATGTACAACATGATGGA; TTAGCCTCGTCGATGAACG	−4~+17; +266~+284	60; 288
SOX2-2	GAAACTTTTGTCGGAGACGGA; ATCATGCTGTAGCTGCCGTT	+237~+257; +502~+521	60; 285
SOX2-3	ACAGTTACGCGCACATGAA; ATGCTGATCATGTCCCGGA	+473~+491; +809~+827	60; 355
SOX2-4	ATGCACCGCTACGACGTGA; CCAAAAAGAAGTCCAGGATC	+589~+607; +1190~+1209	60; 621

AT, annealing temperature; PS, product size.
[a]The coding sequence for SOX2: +1~+951.

previously (8, 9). In brief, expression vectors for *SOX2* (WT and MT), chicken *Pax6*, and mouse *Brn2* were generated by inserting corresponding cDNAs into pCMV/SV2 vector (8), and luciferase reporter constructs were created by inserting DC5 and Nes30 into pδ51LucII vector with the δ1-crystallin promoter (8, 16). Subsequently, chicken embryo liver cells were transfected with (1) the reporter vector containing DC5 and the expression vectors for SOX2 (WT, MT, and WT plus MT) and Pax6, and (2) the reporter vector containing Nes30 and the expression vectors for SOX2 (WT, MT, and WT plus MT) and Brn2, together with phRG-TK vector (Promega) used as an internal control for the transfection. Luciferase assays (≥3 times) were performed at 48 h after the transfection.

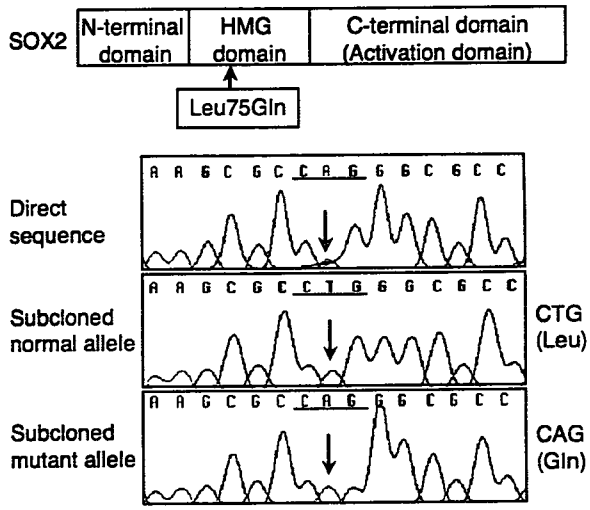

Figure 1 Mutation analysis of *SOX2* showing a heterozygous missense mutation (224T>A, L75Q). The mutation has been indicated by the direct sequencing, and confirmed by the subsequently performed sequencing of the subcloned normal and mutant alleles. The L75Q mutation resides at the HMG (high mobility group) domain with a DNA-binding capacity.

Results

Mutation analysis

A heterozygous transversion (224T>A) resulting in a substitution of the 75th leucine codon with a glutamine codon (L75Q) was identified in the helix II of DNA-binding HMG domain (Fig. 1). This missense mutation was absent in the 100 control subjects.

DNA-binding assay

The binding affinity of the MT SOX2 protein was severely reduced, as compared with that of the WT SOX2 protein (Fig. 2). Since 5 ng of the MT SOX2 protein gave similar intensity of the shifted band to 0.5 ng of the WT SOX2 protein, the DNA-binding affinity of the MT SOX2 protein was assessed as approximately 10% of that of the WT SOX2 protein.

Transactivation analysis

The transactivation potential of the MT SOX2 protein was markedly decreased, as compared with that of the WT SOX2 protein (approximately 20% for DC5 and approximately 30% for Nes30; Fig. 3A and B). Consistent with the severely attenuated (approximately 10%) DNA-binding affinity, the activation levels were similar between the assays using 2 ng of the MT SOX2 expression vector and those using 0.2 ng of the WT SOX2 expression vector, for both reporters with DC5 and Nes30. Furthermore, the activation levels were comparable between the assays with WT SOX2 protein only and those with WT plus MT SOX2 proteins for both reporters, indicating the lack of a dominant negative effect of the MT SOX2 protein.

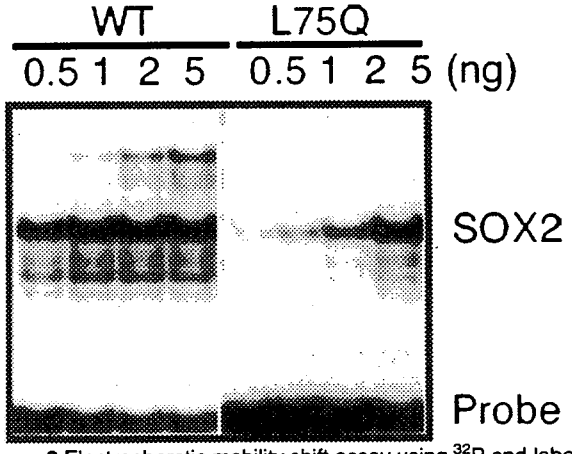

Figure 2 Electrophoretic mobility shift assay using ^{32}P end-labeled DC5 and the wildtype (WT) and the L75Q mutant SOX2 proteins.

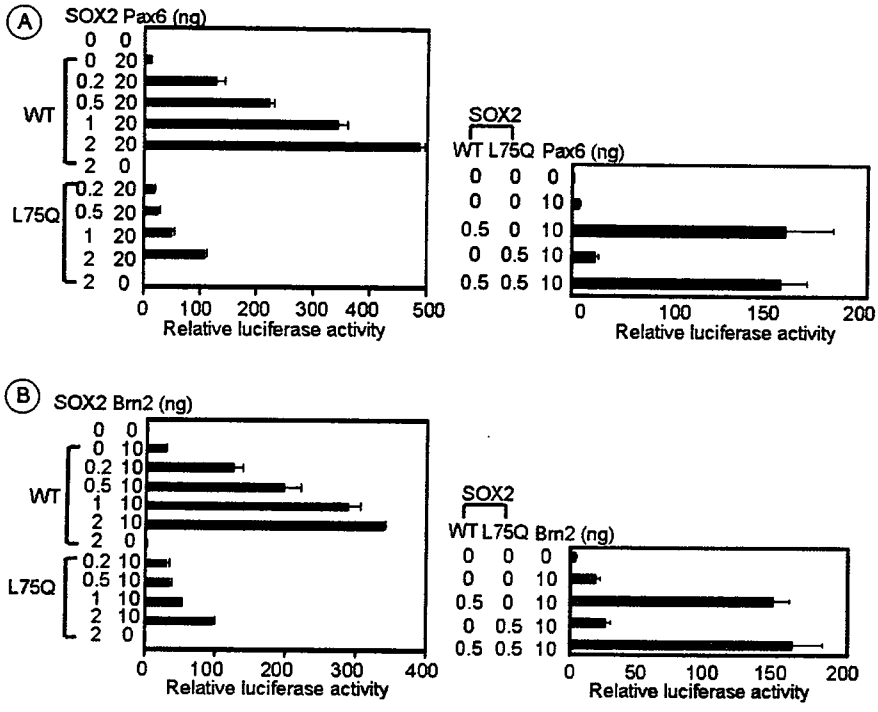

Figure 3 Transactivation function of the wildtype (WT) and the L75Q mutant SOX2 proteins for DC5 (A) and Nes30 (B). Relative luciferase activities are shown with the mean and the S.D., with an activity generated by the reporter lacking DC5 or Nes30 being taken as 1.

Discussion

A heterozygous L75Q mutation was identified in the HMG domain of *SOX2* in this patient. Furthermore, the MT SOX2 protein was shown to have reduced DNA-binding affinity and decreased transcription activity with no dominant negative effect. These findings suggest that the L75Q mutation is a hypomorphic mutation retaining a residual activity.

This patient had unilateral anophthalmia and apparently normal mental development. This mild phenotype would primarily be due to the residual SOX2 activity. In addition, other genetic and environmental factors would also be relevant to the phenotypic consequences, because the ocular and CNS phenotype is not necessarily dependent on the residual activity alone (12). Furthermore, the degree of residual SOX2 activity and the status of other factors would also be involved in the development of several infrequent features such as esophageal atresia, sensorineural deafness, and short stature (10–12, 17, 18).

The salient feature of this patient is isolated HH. This provides further support for *SOX2* being involved in the regulation of gonadotropin production. In this context, several findings are noteworthy. First, she had obvious HH in the presence of relatively mild ocular lesion and apparently normal CNS function. This would primarily be compatible with the phenotype of heterozygous *Sox2* knockout mice exhibiting pituitary dysfunction and normal ocular development (12), and may suggest that gonadotropin production is more sensitive to the reduced SOX2 function than ocular and CNS development. Second, there was no anterior pituitary hypoplasia. This implies that *SOX2* mutations do not necessarily lead to pituitary hypoplasia. Third, other pituitary hormones were normal, as in the patients described by Kelberman *et al.* (12). This suggests that the gonadotropin is most vulnerable to reduced *SOX2* dosage among pituitary hormones. However, it should be pointed out that heterozygous *Sox2* knockout mice have multiple pituitary hormone deficiency (12), and that SOX2 protein is capable of transactivating *HESX1* (12, 19), a causative gene for panhypopituitarism and optic nerve abnormality (20). Thus, in conjunction with anterior pituitary hypoplasia in most patients with *SOX2* mutations (12), heterozygous *SOX2* mutations could affect other pituitary hormones in exceptional patients.

It remains to be clarified how *SOX2* mutations lead to HH. However, it has been reported that murine *Sox2* expression is identified in the presumptive hypothalamus and the Rathke's pouch at E11.5, whereas it is confined around the Rathke's pouch lumen at a later age and detected in some non-endocrine cells only in the adult pituitary (12). Thus, HH in *SOX2* mutations may primarily be ascribed to dysregulated hypothalamopituitary axis during the early life, rather than a cell-type specific dysfunction of the gonadotropes.

In summary, the results provide further support for the positive role of *SOX2* in the regulation of gonadotropin production. Further studies will permit to define the pituitary phenotype and the molecular mechanism leading to HH in patients with *SOX* mutations.

Acknowledgements

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Haplotype analysis of the estrogen receptor 1 gene in male genital and reproductive abnormalities

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BACKGROUND: We have recently suggested that homozygosity for a specific 'AGATA' haplotype within a ~50 kb linkage disequilibrium (LD) block of the gene for estrogen receptor α (*ESR1*) may raise the susceptibility to cryptorchidism by enhancing estrogenic effects of environmental endocrine disruptors (EEDs). METHODS: Haplotype analysis of *ESR1* was performed in 328 Japanese subjects, i.e. 70 patients with micropenis (MP), 43 patients with hypospadias (HS), 80 patients with spermatogenic failure (SF) and 135 control males. Genotyping was performed by the 5′ nuclease assay. RESULTS: The LD block was identified in each of the patient groups and in the control males. The frequency of homozygotes for the specific 'AGATA' haplotype was markedly higher in the HS patients [$P = 0.0000033$, odds ratio [OR] = 11.26] and slightly higher in the MP patients ($P = 0.034$, OR = 3.64) than in the control males, and the 'AGATA' haplotype was strongly associated with HS ($P = 0.0000022$, OR = 11.26) and weakly associated with MP ($P = 0.040$, OR = 3.64) in a recessive mode. There was no significant difference between the SF patients and the control males. CONCLUSIONS: Our results support the hypothesis that homozygosity for the specific *ESR1* 'AGATA' haplotype may increase the susceptibility to the development of male genital abnormalities in response to estrogenic EEDs.

Key words: environmental endocrine disruptors/estrogen receptor 1/haplotype analysis/susceptibility/undermasculinization

Introduction

The prevalence of cryptorchidism (CO), hypospadias (HS), spermatogenic failure (SF) and testicular cancer has gradually increased during the last few decades at least in several countries (Toppari *et al.*, 1996; Hutson *et al.*, 1997; Kurzrock and Karpman, 2004) and that of micropenis (MP) also appears to have increased (Toppari and Skakkebaek, 1998). Such deterioration of male genital and reproductive health has also been observed in many wildlife species (Toppari *et al.*, 1996; McLachlan, 2001). Because of the rapid pace, this phenomenon is primarily ascribed to alteration of a variety of environmental or lifestyle factors including environmental endocrine disruptors (EEDs) sedentary work, and obesity (Toppari *et al.*, 1996; Hutson *et al.*, 1997; McLachlan, 2001; Skakkebaek *et al.*, 2001; Kurzrock and Karpman, 2004; Kishi *et al.*, 2005; Magnusdottir *et al.*, 2005). For EEDs, estrogenic effects exerted by most, not all, EEDs may play a major role in this phenomenon, because exposure to estrogenic agents is known to result in various male genital and reproductive abnormalities (Stillman, 1982; Wilcox *et al.*, 1995; Toppari *et al.*, 1996; Nef *et al.*, 2000; McLachlan, 2001; Klip *et al.*, 2002; Kim *et al.*, 2004).

The effects of EEDs, if they indeed exist, would depend on the genetic susceptibility, in addition to the dosage and character of exposed EEDs and the developmental stage of EEDs exposure. For estrogenic EEDs, genetic susceptibility would primarily be ascribed to variations of the genes for estrogen receptor (ER), because estrogenic effects of EEDs are primarily mediated by ER (Toppari *et al.*, 1996; McLachlan, 2001). Indeed, estrogenic EEDs can bind to both ER(encoded by *ESR1* and ER(encoded by *ESR2* with variable affinities (McLachlan, 2001).

[8]*The first and the second authors equally contributed to this work.*

Thus, we have previously performed a haplotype analysis of *ESR1* in 63 Japanese patients with CO and 47 control males with normal external genitalia, using 15 single nucleotide polymorphisms (SNPs 1–15) that are widely distributed throughout >300 kb genomic sequence of *ESR1* (Yoshida *et al.*, 2005). Haplotype is a list of alleles on a single chromosome, and alleles at loci within a linkage disequilibrium (LD) block can be inherited as a unit because of lack of a recombination (Terwilliger and Ott, 1994). Thus, when a significant association is identified between a disease phenotype and a specific haplotype within a LD block, a susceptibility allele(s) is expected to reside on the haplotype-specific sequence within the block (Davidson, 2000).

We identified a significant association of CO with homozygosity for a specific 'AGATA' haplotype within an ~50 kb LD block spanning SNPs 10–14 in the 3′ region of *ESR1* (Yoshida *et al.*, 2005). This may suggest the involvement of genetic susceptibility to estrogenic EEDs in the development of CO, although there is no direct evidence for an association between estrogenic EEDs and CO. Here, we examined whether the specific *ESR1* 'AGATA' haplotype is also associated with various male genital and reproductive abnormalities.

Materials and methods

Subjects

We studied a total of 193 Japanese male patients, consisting of 70 MP patients aged 0–13 (median 6.0 years) (33 with mild MP, from −2.1 to −2.5 SD, and 37 with severe MP, below −2.5 SD), 43 HS patients aged 0–27 (median 7.0 years) [18 with mild glandular ($n = 5$) or penile ($n = 13$) HS and 25 with severe scrotal ($n = 15$) or perineal ($n = 10$) HS] and 80 SF patients aged 32–52 (median 41.0 years) [69 azoospermic and 11 oligozoospermic (sperm count $<20 \times 10^6$/mL): 46 biopsied (38 with Sertoli cell-only phenotype and 8 with maturation-arrest phenotype) and 34 non-biopsied]. MP was diagnosed on the basis of the age-matched Japanese reference data (Fujieda and Matsuura, 1987). HS was diagnosed by physical examination and was surgically treated in all the patients. SF was demonstrated by repeated (two or more times) analyses of semen samples obtained after 5–7 days of abstinence, after excluding hypogonadotropic hypogonadism, seminal tract obstruction, varicocele, retrograde ejaculation and history of CO and mumps orchiditis. None of the MP and SF patients had HS, and 12 of the HS patients manifested apparent MP (the precise penile lengths had not been measured in most of the HS patients).

All the patients satisfied the following selection criteria: (i) lack of extragenital anomalies, (ii) 46,XY karyotype in all the ≥20 lymphocytes analysed, (iii) no significant expansion of CAG repeat length at exon 1 of the androgen receptor (*AR*) *gene* that is known to raise the susceptibility to male genital and reproductive abnormalities (Dowsing *et al.*, 1999; Lim *et al.*, 2000) and (iv) no demonstrable mutation of *AR* and *SRD5A2*. In addition, no Y chromosomal microdeletion was detected by the PCR analysis for 36 loci including *RBMY* and *DAZ* in the HS and SF patients. The methods and results of *AR* and *SRD5A2* analyses and those of Y chromosomal deletion analysis have been reported previously (Tateno *et al.*, 2000; Ishii *et al.*, 2001; Muroya *et al.*, 2001; Sasagawa *et al.*, 2001; Itoh *et al.*, 2002; Sasaki *et al.*, 2003), except for the unpublished results of *SRD5A2* analysis in the HS patients and those of Y chromosomal deletion analysis in the SF patients (performed by Ogata and Sasagawa). Unfortunately, although serum estrogen values are considered to be an important factor, they were not measured in these patients.

We also examined a total of 135 Japanese control males, consisting of 82 control boys with normal external genitalia aged 4–16 (median 8.5 years), including the previously reported 47 subjects (Yoshida *et al.*, 2005), and 53 control adult males with proven fertility aged 24–50 (median 35.5 years), after obtaining permission. The control boys were seen because of short to low-normal stature (−1.5 to −3.0 SD) and were found to have no discernible abnormality by cytogenetic, skeletal and endocrine studies. The control adult males were normal in height (−2.0 SD to +2.0SD).

SNP analysis

This study was approved by the Institutional Review Board Committees at National Center for Child Health and Development, Keio University Hospital, and Yamagata University Hospital, and informed consent was obtained from each subject or the parent(s). The SNPs 8–15 covering the LD block identified in the previous study (Yoshida *et al.*, 2005) were analysed using leukocyte genomic DNA of each subject (Figure 1 and Table I). Genotyping was performed by the 5′ nuclease assay on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) (De La Vega *et al.*, 2002). Pearson's χ^2-test with one degree of freedom was applied to test whether the genotyping data of each SNP are in the Hardy-Weinberg equilibrium (http://en.wikipedia.org/wiki/Hardy-Weinberg).

Haplotype analysis

We first examined whether the LD block spanning SNPs 10–14 detected in our previous study (Yoshida *et al.*, 2005) could be identified in the patients and the control males. Haplotype inference was performed by the maximum likelihood method using expectation-maximization algorithm (Excoffier and Slatkin, 1995) implemented in the software LDSUPPORT (Kitamura *et al.*, 2002). A pairwise $|D'|$ value (the absolute value for the disequilibrium parameter) that ranges from 0 (complete linkage equilibrium status) to 1.0 (complete LD status) was estimated by the method of Terwilliger and Ott (1994). A haplotype block was determined by the method of Zhu *et al.* (2003) using the software developed by Kamatani *et al.* (2004).

Statistical significance of the differences in estimated haplotype frequencies and homozygote frequencies was examined using the R environment (http://www.r-project.org/), together with odds ratio (OR) and the 95% confidence interval. Association of each estimated haplotype with disease phenotype as well as its OR was analysed by PENHAPLO software (Ito *et al.*, 2004) that tests the difference in frequencies of diplotype configurations (combination of two haplotypes in a subject) in a dominant mode (comparison of the frequencies of subjects with one risk haplotype between cases and controls) and in a recessive mode (comparison of the frequencies of subjects with two risk haplotypes between cases and controls). A $P < 0.05$ was considered statistically significant.

Results

SNP analysis

The genotype frequencies of SNPs 8–15 were in accord with the Hardy–Weinberg equilibrium, except for the increased 'AA' genotype frequencies of SNP 10 ($P = 0.023$) and SNP 12 ($P = 0.030$) in the HS patients. The raw genotyping data and the results of statistical analysis for the allele and genotype frequencies of each SNP are available on request.

Haplotype analysis

The pairwise LD maps are shown in Figure 1. The LD block spanning SNPs 10–14 was identified in the patients and the

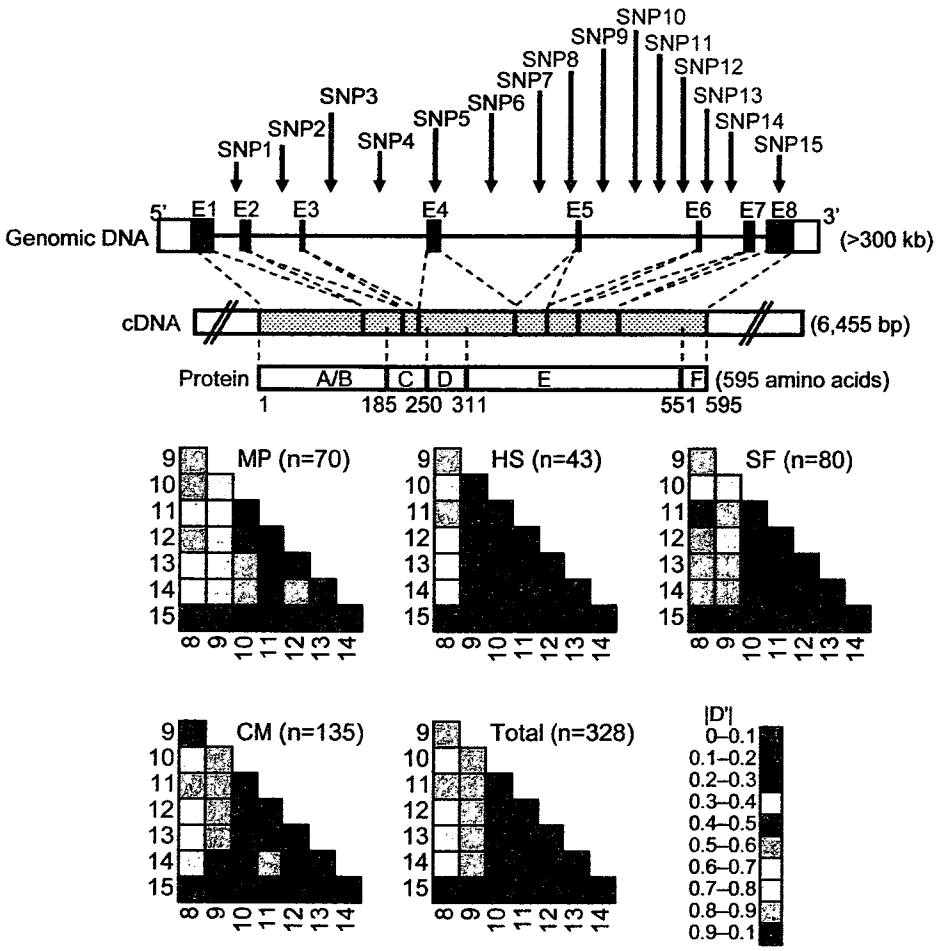

Figure 1. (Upper part) Physical positions of single nucleotide polymorphisms (SNPs) 8–15 examined in the present study, together with those of SNPs 1–7 which have also been analysed in our previous study of cryptorchidism (Yoshida *et al.*, 2005). The estrogen receptor 1 (*ESR1*) gene consists of eight exons (E1–E8) and extends over a distance of 300 kb in physical length (Shupnik, 2002). The coding regions are shown as solid boxes, and the 5′- and the 3′-untranslated regions are depicted as open boxes. The *ESR1* complementary DNA is 6455 bp long and is translated into a 595 amino acid protein that is divided into six common series of structural regions referred to as A to F. The A/B region harbours the ligand independent transactivation domain, the C region harbours the DNA-binding domain and the E region carries the ligand-binding domain and the ligand-dependent transactivation domain. (Lower part) Pairwise linkage disequilibrium maps. MP, micropenis patients; HS, hypospadias patients; SF, spermatogenic failure patients; CM, control males; and $|D'|$; an absolute value for the disequilibrium parameter.

Table I. SNPs 8–15 examined in the present study

SNP	NCBI No.	Position (NT_025741 sequence)[a]	Type (AF)	Remark
SNP 8	rs6905370	Intron 4 (152,367,890 bp)	G/A (56%/44%)	
SNP 9	rs13203975	Intron 5 (152,374,797 bp)	G/A (80%/20%)	
SNP 10	rs926779	Intron 5 (152,397,613 bp)	G/A (69%/31%)	
SNP 11	rs3020364	Intron 5 (152,408,811 bp)	A/G (56%/44%)	
SNP 12	rs6932902	Intron 5 (152,418,217 bp)	G/A (77%/23%)	
SNP 13	rs3020371	Intron 6 (152,425,513 bp)	C/T (58%/42%)	
SNP 14	rs3020375	Intron 6 (152,431,661 bp)	C/A (55%/45%)	
SNP 15	rs2228480	Exon 8 (152,461,788 bp)	G/A (83%/17%)	Thr594Thr

SNP, single nucleotide polymorphism; NCBI, National Center for Biotechnology Information; AF, allele frequency in 135 control subjects.
[a]According to the NCBI database indicating the standard nucleotide position from the 6p telomere.

control males, with the $|D'|$ value being >0.8 for all the pairs of SNPs 10–14. In particular, the LD block was evident in the HS and SF patients and extended to SNP 9 in the HS patients. Furthermore, when all the patients and the control males were combined, the LD block encompassing SNPs 10–14 was clearly identified, with the $|D'|$ value being >0.95 for all the pairs of SNPs within the LD block except for the $|D'|$ value of 0.93 between SNPs 11 and 14.

Within the LD block, the specific 'AGATA' haplotype was identified in the MP, HS and SF patients and in the control

males as the second most frequent haplotype, together with three other haplotypes ('GAGCC', 'GGGTA', and 'AGGTA'). The frequency of the 'AGATA' haplotype was significantly higher in the HS patients than in the control males and that of the 'AGATA' homozygotes was markedly higher in the HS patients and mildly higher in the MP patients than in the control males; consistent with this, the 'AGATA' haplotype was strongly associated with HS phenotypes and weakly associated with MP phenotype in a recessive mode (Tables II and III). No significant difference was identified in the comparisons between the SF patients and the control males, as well as between subgroups of patients (e.g. the frequency of 'AGATA' homozygotes: mild versus severe MP, 3/33 versus 4/37, respectively, $P = 0.81$; mild versus severe HS, 4/18 versus 7/25, respectively, $P = 0.67$; azoospermia versus oligozoospermia, 5/69 versus 1/11, respectively, $P = 0.83$; biopsied versus non-biopsied SF, 4/46 versus 2/34 respectively, $P = 0.64$; and Sertoli cell-only phenotype versus maturation-arrest phenotype, 1/8 versus 3/38, respectively, $P = 0.67$).

For the remaining three haplotypes, no significant difference was identified for the haplotype and homozygote frequencies and for the association of haplotype with phenotype. The results are available on request.

Discussion

The ~50 kb LD block at the 3′ region of *ESR1* was commonly identified in the patients and the control males. Furthermore, four major estimated haplotypes were predominantly detected for the LD block encompassing SNPs 10–14. These findings suggest that the LD block and the four major haplotypes are well preserved in the Japanese population. Furthermore, according to the International HapMap Project (http://www.hapmap.org/), this LD block also appears to be present in various populations.

Homozygosity for the 'AGATA' haplotype was significantly more frequent in the HS patients and to a lesser extent in the MP patients than the controls. Consistent with this, the 'AGATA' haplotype was strongly associated with HS and weakly associated with MP in a recessive mode. These findings provide further support for the previously proposed notion that homozygosity for the 'AGATA' haplotype may raise the susceptibility to undermasculinized genitalia in response to estrogenic EEDs (Yoshida *et al.*, 2005). In this regard, although the genotyping data of SNPs 10 and 12 in the HS patients did not follow the Hardy–Weinberg equilibrium, with increased 'AA' genotypes, this would be regarded as a reflection of the strong association between HS and the homozygosity for the 'AGATA' haplotype. Indeed, the 'A' allele of SNP 10 is nearly specific and that of SNP 12 is completely specific to the 'AGATA' haplotype.

The specific 'AGATA' haplotype may enhance the ESR1 signaling, facilitating the development of HS and MP as well as CO. Indeed, maternal exposure to estrogenic agents is known to cause HS and CO in the human and the rodents (Stillman, 1982; Nef *et al.*, 2000; Klip *et al.*, 2002; Kim *et al.*, 2004), although there are no data on MP. In this regard, several matters are noteworthy: (i) excessive estrogenic effects not only affect androgen production for external genital development by reducing gonadotropin secretion and testicular steroidogenic enzyme activity (O'Donnell *et al.*, 2001; Shupnik, 2002), but also repress *INSL3* expression for the gubernacular development (Nef *et al.*, 2000) (it may also disturb the focal endocrine environment such as the androgen/estrogen ratio in the external genital tissues) (Toppari and Skakkebaek, 1998; Dietrich *et al.*, 2004); (ii) impaired androgen effects around the critical period for sex development usually result in structurally abnormal external genitalia including HS, whereas those after the critical period usually lead to MP and/or CO without structural abnormalities (Grumbach *et al.*, 2002) and (iii) the estrogenic effects of EEDs should be persistent including the critical period, and the sensitivity to EEDs could be higher in the fetal life, especially around the critical period (Wilcox *et al.*, 1995; McLachlan, 2001; Kurzrock and Karpman, 2004).

Table II. Frequency of the 'AGATA' haplotype (SNPs 10–14)

	Haplotype	Homozygote
MP ($n = 70$)	30.0% (42/140)	7/70
HS ($n = 43$)	41.9% (36/86)	11/43
SF ($n = 80$)	28.1% (45/160)	6/80
CM ($n = 135$)	21.9% (59/270)	4/135

MP, patients with micropenis; HS, patients with hypospadias; SF, patients with spermatogenic failure; CM, control males.

Table III. Summary of the statistical analysis of the AGATA haplotype (SNPs 10–14)

		Comparison of the frequency		Assocation with phenotype	
		Haplotype	Homozygosity	Dominant model[a]	Recessive model[b]
MP versus CM	*P*-value	0.069	0.034	0.14	0.040
	OR (95% CI)	1.53 (0.97–2.43)	3.64 (1.02–12.9)	1.54 (0.86–2.77)	3.64 (1.06–14.3)
HS versus CM	*P*-value	0.00026	0.0000033	0.051	0.0000022
	OR (95% CI)	2.57 (1.53–4.30)	11.26 (3.36–37.67)	2.02 (0.98–4.11)	11.26 (3.60–42.8)
SF versus CM	*P*-value	0.14	0.13	0.25	0.13
	OR (95% CI)	1.39 (0.89–2.19)	2.63 (0.73–9.71)	1.38 (0.79–2.42)	2.66 (0.74–10.7)

OR, odds ratio; CI, confidence interval.
[a]Homozygotes plus heterozygotes versus non-carriers for the examined haplotype.
[b]Homozygotes versus heterozygotes plus non-carriers for the examined haplotype.

Our findings suggest that the specific haplotype may raise the susceptibility to HS primarily because of reduced androgen effects around the critical period and to CO primarily because of impaired INSL3 effects after the critical period. In addition, since it is unlikely that reduced androgen effects become obvious after the critical period, this would explain why the association between the specific haplotype and MP remained mild. It should be pointed out, however, that the MP patients were few in number. Thus, a more obvious association may be identified between the specific haplotype and MP, if a larger number of MP patients are analysed.

In contrast, no significant association was identified between the 'AGATA' haplotype and SF, although genetic susceptibility may be relevant to the development of SF in response to estrogenic EEDs (Toppari *et al.*, 1996). Indeed, exposure to estrogenic agents is known to result in SF (O'Donnell *et al.*, 2001). However, in contrast to abnormal external genitalia that develop during the fetal life, SF becomes discernible in adulthood and, therefore, could be influenced by multiple genetic and environmental factors for a long time. In addition, the prevalence of HS appears to have increased (Kishi *et al.*, 2005), whereas that of SF may have remained unchanged during the last few decades in Japan (Itoh *et al.*, 2001). Thus, the involvement of genetic susceptibility to estrogenic EEDs in the development of SF seems obscure in contemporary Japanese males, although it may become clearer in the future. Alternatively, a variant(s) of *ESR2* may play an important role in the development of SF in response to EEDs, because *ESR2* is clearly expressed in fetal and adult testes, especially in germ cells (O'Donnell *et al.*, 2001). Indeed, an association of *ESR2* polymorphisms with SF as well as HS has been reported recently (Aschim, *et al.*, 2005; Beleza-Meireles, *et al.* 2006). In addition, variations in serum estrogens, though not measured here, may also be relevant to the lack of association.

In summary, the present study suggests the involvement of genetic susceptibility in the development of external genital abnormalities, and this may be in response to estrogenic EEDs. However, several points should be made in this study. First, although all the patients and control males were Japanese, this does not exclude a possible contribution of ethnic differences to the positive results. Indeed, the Japanese are derived from at least two different ancestral populations (Hammer *et al.*, 2006). Second, the number of analysed subjects is small, and the present study focused on the LD block region rather than the whole *ESR1* gene. Third, it remains to be determined whether the positive results for undermasculinization can be reproduced in other countries or populations with an increased prevalence of male genital abnormalities, whether the specific *ESR1* haplotype is truly not associated with SF and whether an *ESR2* variant(s) is involved in the susceptibility to male genital and reproductive abnormalities. Lastly, the notion concerning EEDs is still conceptual, and other environmental or lifestyle factors are also likely to be involved in the deterioration of male genital and reproductive health (Skakkebaek *et al.*, 2001; Magnusdottir *et al.*, 2005). Thus, further studies are necessary to clarify the relevance of genetic susceptibility to the male genital and reproductive abnormalities in response to EEDs.

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GENETICS

Silver-Russell syndrome in a girl born after *in vitro* fertilization: partial hypermethylation at the differentially methylated region of *PEG1/MEST*

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Abstract *Purpose:* The prevalence of low birth weight (LBW) is increased in subjects born after assisted reproduction technology (ART), and defective imprinting has frequently been identified in patients with Beckwith-Wiedermann and Angelman syndromes conceived by ART. Thus, we examined methylation pattern in a girl born after ART who had Silver-Russell syndrome (SRS) which can be caused by maternal uniparental disomy for chromosome 7 and by hypomethylation of the differentially methylated region (DMR) of *H19*.

Methods: We examined methylation status of 31 cytosines at the CpG dinucleotides in the DMR of *PEG1/MEST* on 7q32.2 and 23 cytosines at the CpG dinucleotides in the DMR of *H19* on 11p15, using leukocyte genomic DNA.

Results: Eight of the 31 cytosines in the patient and four of the 31 cytosines in the father were hypermethylated in the *PEG1/MEST*-DMR. In the *H19*-DMR, no abnormal methylation pattern was identified in the patient.

Conclusion: The results suggest that hypermethylation of paternally expressed genes including *PEG1/MEST*, which usually have growth-promoting effects, may be relevant to LBW in subjects conceived by ART.

Partial hypermethylation was identified at the differentially methylated region of paternally expressed *PEG1/MEST* in a girl with Silver-Russell syndrome born after *in vitro* fertilization.

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Introduction

Silver-Russell syndrome (SRS) is a congenital developmental disorder characterized by pre- and postnatal growth failure, body asymmetry, relative macrocephaly, triangular face, and fifth-finger clinodactyly [1]. Precocious puberty is also occasionally observed in this condition. Since maternal uniparental disomy for chromosome 7 (mUPD7) has been identified in 7–10% of patients with SRS, this implies the involvement of a single or plural imprinted genes on chromosome 7 in the development of SRS [1]. The possibility of unmasking of a recessive allele(s) by isodisomy is unlikely, because both isodisomy and heterodisomy have been found in SRS with no common isodisomic region [2]. Furthermore, molecular studies in several key patients have suggested two separate candidate regions for SRS, 7p11.2–p13 [3] and 7q31–qter [4], and several candidate genes such as *GRB10* (growth factor receptor-bound protein 10) on 7p12 and *PEG1/MEST* (paternally expressed gene 1/mesoderm-specific transcript) on 7q32.2 have been identified [1], although the gene(s) responsible for SRS has not been identified to date. In addition, recent studies have shown hypomethylation of the differentially methylated region (DMR) located upstream of *H19* (*H19*-DMR) in a considerable fraction of SRS patients, providing further support for the relevance of imprinting failure in SRS [5].

In vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) has become a widely utilized method for infertility. However, such assisted reproduction technology (ART) has been regarded as a risk factor for low birth weight (LBW) and major birth defects [6, 7].

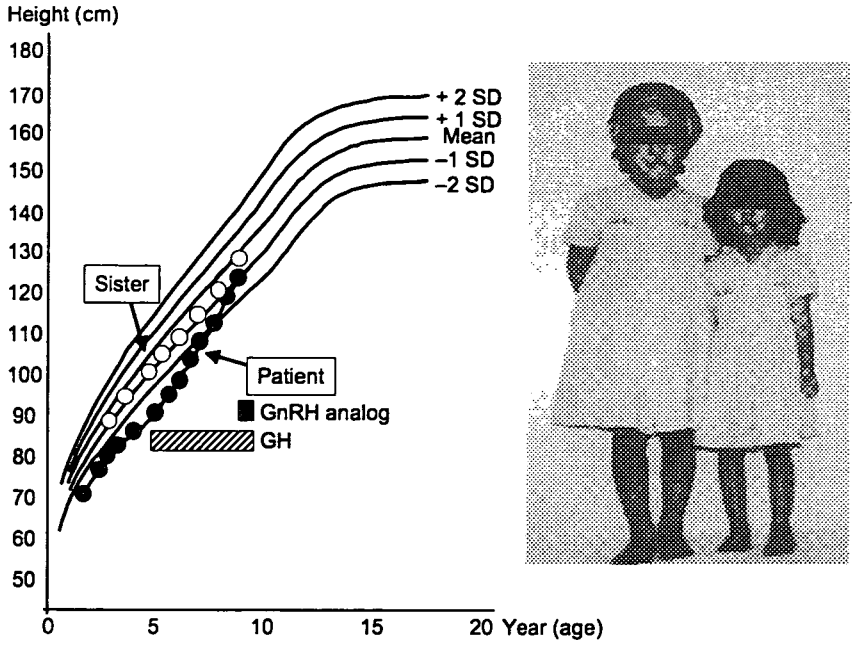

Fig. 1 Growth charts depicted on the standard growth curves for the Japanese girls and photographs of the twin sisters at 4 11/12 of age. GH: growth hormone; and GnRH: gonadotropin releasing hormone

Furthermore, recent studies in two human imprinting disorders, Beckwith-Wiedermann syndrome and Angelman syndrome, have indicated aberrant methylation pattern in most of the affected children born after ART, suggesting that ART may perturb the epigenetic imprinting process [8–10]. In addition, the prevalence of Beckwith-Wiedermann syndrome has been reported to be significantly higher in children conceived by ART than in those conceived naturally [11].

To our knowledge, however, abnormal methylation pattern in SRS patients born after ART has been reported in only a single patient who was conceived by ICSI and found to have hypomethylation of the *H19*-DMR [12]. Here, we report on methylation analysis in a girl with SRS born after IVF.

Materials and methods

Case report

This Japanese girl and her twin sister were conceived with use of IVF, and delivered by a cesarean section at 37 weeks of gestation. The parents were non-consanguineous and clinically normal. The paternal height was 174.0 cm (+0.6 SD), and the maternal height 150.0 cm (−1.6 SD). They had a male infant after natural conception in the second year of their conjugal life, who had severe hydrocephalus and deceased at six months of age. Thereafter, they failed to conceive for 12 years, and received IVF. Maternal oocytes taken after gonadotropin stimulation were mixed with paternal sperms collected using a condom. Six fertilized ova were obtained, and two of them were transferred to the maternal uterus, resulting in a production of the twin sisters.

At birth, her length was 36.5 cm (−6.0 SD), weight 1.25 kg (−4.6 SD), and head circumference (HC) 30.0 cm (−2.0 SD). At 3 6/12 years of age, she was seen because of short stature. Her height was 82.1 cm (−3.6 SD), weight 7.7 kg (−3.9 SD), and HC 46.2 cm (−1.5 SD). The growth chart and photograph are shown in Fig. 1. Physical examination showed SRS-compatible features such as triangle face with micrognathia, relative macrocephaly, high arched palate, right hemihypotrophy, and bilateral clinodactyly of the 5th fingers. There were no discernible major anomalies. Psychomotor development was age-appropriate. Endocrine studies for short stature were normal, as were radiological studies. Her karyotype was 46,XX in all the 20 lymphocytes examined. She received growth hormone therapy from 4 11/12 to 8 7/12 years of age, and showed upward growth shift. She exhibited breast development from 7 1/12 years of age and pubic hair development from 8 2/12 years of age, and had menarche at 8 8/12 years of age (menarchial age of normal Japanese girls: 12.25 ± 1.25 years). She was diagnosed as having central precocious puberty by a gonadotropin releasing hormone (GnRH) test, and was placed on GnRH analog therapy from 8 9/12 years of age. On the last examination at 9 2/12 years of age, her height was 125.5 cm (−1.0 SD), weight 22.3 kg (−1.2 SD), and HC 50.3 cm (−1.5 SD).

The twin sister was clinically normal (Fig. 1). Her birth length was 43.0 cm (−3.1 SD), weight 2.3 kg (−2.0 SD), and HC 31.3 cm (−1.0 SD). She showed catch-up growth during infancy, and her height remained around −0.8 SD in childhood. At 9 2/12 years of age, he exhibited breast