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省略

G. 知的財産権の出願・登録状況

該当なし

平成 26 年度厚生労働科学研究委託費（難治性疾患等克服研究事業
（難治性疾患等実用化研究事業（難治性疾患実用化研究事業））
分担研究報告書

インプリンティング異常症発症および合併症発症メカニズムの解明：患者由来iPS細胞を用いての研究
研究統括、検体収集、遺伝子診断、患者検体・iPS細胞・分化誘導細胞の解析

研究分担者　鏡雅代　　国立成育医療研究センター研究所分子内分泌研究部室長

研究要旨

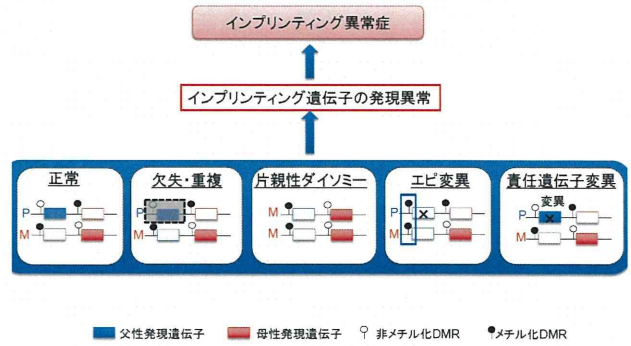
希少疾患であるインプリンティング異常症の病態解明を目的に、我々が開発したインプリンティング異常症遺伝子診断システムを用いて8インプリンティング異常症表現型患者の遺伝子診断を約250名で行った。約30%でインプリンティング異常症と遺伝子診断された。加えて、Kagami-Ogata Syndrome エピ変異症例、欠失症例計6名においてiPS細胞樹立の同意を得たのち、手術の際の余剰皮膚片の提供をうけ、これらでiPS細胞を作成した。

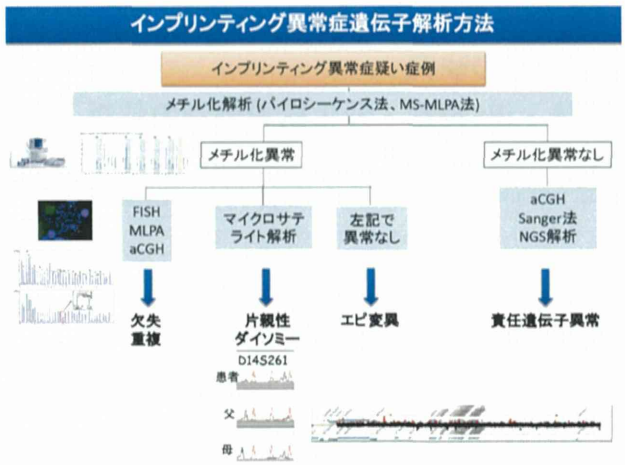
A．研究目的

希少疾患であるインプリンティング異常症は代表的な疾患として下記の 8 疾患がある。

疾患名	責任領域	DMR
Silver-Russell 症候群 (SRS)	11p15 Ch 7	*H19* （Ch 7は不明）
Beckwith-Wiedemann 症候群 (BWS)	11p15	*H19*, Kv
Kagami-Ogata症候群 (KOS14)（旧 UPD(14)pat症候群）	14q32.2	IG, *MEG3*
Temple 症候群 (TS14)（旧 UPD(14)mat症候群）	14q32.2	IG, *MEG3*
Prader-Willi 症候群 (PWS)	15q11-13	*SNRPN*
Angelman 症候群 (AS)	15q11-13	*SNRPN*
新生児一過性糖尿病 (TNDM)	6q24	*ZAC1*
偽性副甲状腺機能低下症 (PHP)	20q13.3	*GNAS* A/B

これらの 8 疾患はともに責任インプリンティング領域のインプリンティング遺伝子発現異常により生じ、その原因としては、片親性ダイソミー、インプリンティング領域を含む欠失、メチル化可変領域（DMR）のメチル化異常であるエピ変異、責任遺伝子変異により生じる。我々は先行研究からこれら 8 疾患の遺伝子診断システムを開発し、学会などを通して全国の臨床医に周知を図ってきた。インプリンティング異常症患者由来 iPS 細胞の作成には、まず患者の遺伝子診断が必須であり、その上で、iPS 細胞樹立に必要な患者皮膚繊維芽細胞の入手および検体採取施設における倫理審査が必要である。





B．研究方法

前頁図のように、我々は代表的な 8 インプリンティング異常症の遺伝子診断法を開発した。その表現型、内分泌学的データなどからインプリンティング異常症が疑われた患者の主治医からの遺伝子解析の依頼に対し、患者の血液から抽出した gDNA を用いて遺伝子解析を行う。具体的には、インプリンティング異常症責任領域のインプリンティングセンターとして機能する 9 か所の DMR のメチル化解析をパイロシーケンス法でスクリーニングする。メチル化異常を同定した症例に対しては、メチル化異常の原因がインプリンティング領域を含む欠失なのかの同定のために aCGH, FISH, MLPA による解析を、その領域の存在する染色体がどちらかの親に由来する片親性ダイソミー (UPD) なのかの判定のためにマイクロサテライトマーカー解析を行い、これらで異常がなく、メチル化異常を認める症例をエピ変異と診断した。責任遺伝子が明らかなものについては変異解析も行った。遺伝子診断された患者の主治医に対して、iPS 細胞作成に必要な患者皮膚繊維芽細胞を入手する機会となる手術の際に皮膚片の採取させてほしい旨依頼し、倫理委員会で本研究の承認ののち皮膚片を採取し、皮膚繊維芽細胞を保存し、iPS 細胞を作成した。

C．研究結果

1.インプリンティング異常症患者の集積

本年度は、8 インプリンティング異常症表現型を持つインプリンティング異常症疑い症例約 150 名および、原因不明の SGA 性低身長の患者約 100 名につき遺伝子解析を行った。SRS, TS14, PWS は SGA 性低身長を特徴とすることから原因不明の SGA 性低身長にインプリンティング異常症が含まれている可能性があるためである。11 番染色体インプリンティング領域が関連する BWS は 6 名遺伝子診断され、BWS と鏡像関係にある SRS の 11 番染色体インプリンティング領域名内の *H19*-DMR の低メチル化を 10 名に、SRS のもう一つの原因である 7 番染色体母親性ダイソミーを 5 名に同定した。15 番染色体インプリンティング異常症である PWS は 13 名で、AS は 3 名で遺伝子診断された。20 番染色体インプリンティング異常症である偽性副甲状腺機能低下症は 23 名で同定された。14 番染色体インプリンティン異常症である TS14 は 2 名、KOS14 は 2 名同定された。我々はこれまでに本年度分も含めて 30 名以上の KOS14 患者を遺伝子診断しているが、うち 6 名において気管切開、胃瘻造設などで手術が必要となり、皮膚片を採取し iPS 細胞作成のための皮膚繊維芽細胞を保存し、これを用いて iPS 細胞を作成した。

2.SRS 表現型を示す TS14, PWS, UPD(6)mat の同定

次に、このシステムを用いて表現型と関連のあるインプリンティング異常症責任領域以外の DMR についてもメチル化解析を行った。これにより非典型例、インプリンティング異常症間のオーバーラップ症例を同定することができた。一例として、代表的な成長障害疾患である SRS 症例 132 名および SRS の診断基準をみたさない SGA 性低身長 80 名を解析した。SRS 症例 132 名中、既知の原因である *H19*-DMR 低メチル化を 39 名、7 番染色体母親性ダイソミー を 12 名で認めた。81 名の原因不明例のうち UPD(14)mat 症候群を 3 例、PWS を 1

例、6 番染色体母親性ダイソミー (UPD(6)mat) を 1 例同定した。原因不明 SGA 性低身長 76 名の解析では 7.9%にインプリンティング異常症を同定した。

D．考察

本年度は約 150 例のインプリンティング異常症および SGA 性低身長 100 名に対しインプリンティング異常症包括的遺伝子診断システムを用いて解析し約 30%でインプリンティグ異常症に合致する異常を同定した。加えて、インプリンティング異常症の診断基準を満たさない SGA 性低身長におけるインプリンティング異常症や、臨床像とは異なるインプリンティング異常症を同定した。以上の結果は我々の開発したインプリンティング異常症診断システムが、オーバーラップ症例、非典型症例の同定に有用であることを示す。遺伝子診断された症例からすぐに iPS 細胞作成のための皮膚片の採取が可能なわけではないが、より多くの患者を遺伝子診断することによりインプリンティング異常症患者由来 iPS 細胞作成のチャンスが増えると考えている。実際、KOS14 エピ変異症例 3 例、欠失症例 3 例において iPS 細胞樹立にむけての皮膚片採取ができた。今後も、主治医と密に連絡をとりつつ、皮膚片採取が可能な症例に対し積極的にコンタクトを取っていく。

E．結論

インプリンティング異常症約 150 名、SGA 性低身長患者約 100 名において包括的インプリンティング異常症遺伝子解析を行い約 30%でインプリンティング異常症の遺伝子診断に至った。これまでおよび本年に遺伝子診断した KOS14 エピ変異 3 例、欠失症例 3 例で iPS 細胞樹立のための皮膚片の採取できた。

F．健康危険情報

なし

G．研究発表

1．論文発表

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H. 知的財産権の出願・登録状況

１．特許取得

なし

２．実用新案登録

なし

３．その他

様式第１９

学　会　等　発　表　実　績

委託業務題目「インプリンティング異常症発症および合併症発症メカニズムの解明：患者由来iPS細胞を用いての研究」

機関名　独立行政法人国立成育医療研究センター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
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Epimutations of the IG-DMR and the MEG3-DMR at the 14q32.2 imprinted region in two patients with Silver-Russell syndrome-compatible phenotype.	Kagami M, Mizuno S, Matsubara K, Nakabayashi K, Sano S, Fuke T, Fukami M,Ogata T.	Eur J Hum Genet. 2014 [Epub ahead of print]	2014	国外
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ARTICLE

Epimutations of the IG-DMR and the *MEG3*-DMR at the 14q32.2 imprinted region in two patients with Silver–Russell Syndrome-compatible phenotype

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Maternal uniparental disomy 14 (UPD(14)mat) and related (epi)genetic aberrations affecting the 14q32.2 imprinted region result in a clinically recognizable condition which is recently referred to as Temple Syndrome (TS). Phenotypic features in TS include pre- and post-natal growth failure, prominent forehead, and feeding difficulties that are also found in Silver–Russell Syndrome (SRS). Thus, we examined the relevance of UPD(14)mat and related (epi)genetic aberrations to the development of SRS in 85 Japanese patients who satisfied the SRS diagnostic criteria proposed by Netchine *et al* and had neither epimutation of the *H19*-DMR nor maternal uniparental disomy 7. Pyrosequencing identified hypomethylation of the *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the *MEG3*-DMR in two cases. In both cases, microsatellite analysis showed biparental transmission of the homologs of chromosome 14, with no evidence for somatic mosaicism with full or segmental maternal isodisomy involving the imprinted region. FISH and array comparative genomic hybridization revealed neither deletion of the two DMRs nor discernible copy number alteration in the 14q32.2 imprinted region. Methylation patterns were apparently normal in other six disease-associated DMRs. In addition, a thorough literature review revealed a considerable degree of phenotypic overlap between SRS and TS, although body asymmetry was apparently characteristic of SRS. The results indicate the occurrence of epimutation affecting the IG-DMR and the *MEG3*-DMR in the two cases, and imply that UPD(14)mat and related (epi)genetic aberrations constitute a rare but important underlying factor for SRS.
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INTRODUCTION

Human chromosome 14q32.2 harbors an imprinted region with several paternally expressed genes such as *DLK1* and *RTL1* and maternally expressed genes such as *MEG3* and *RTL1as*, together with the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the post fertilization-derived secondary *MEG3*-DMR.[1,2] Consistent with this, maternal uniparental disomy 14 (UPD(14)mat) results in clinically discernible features such as pre- and post-natal growth failure, characteristic face with prominent forehead and micrognathia, small hands, muscular hypotonia, and precocious puberty.[3] These UPD(14)mat clinical features are also caused by microdeletions involving paternally derived *RTL1* and/or *DLK1* and by epimutation (hypomethylation) affecting the normally methylated IG-DMR and *MEG3*-DMR of paternal origin.[2,4–7] Recently, such a clinically recognizable condition has been referred to as 'Temple Syndrome' (TS).[8]

Clinical features of TS partially overlap with those of other imprinting disorders. Indeed, pre- and post-natal growth failure, small hands, and hypotonia during early infancy are also observed in Prader–Willi Syndrome (OMIM 176270),[9] and UPD(14)mat and epimutations involving the IG-DMR and the *MEG3*-DMR have been identified in several patients diagnosed as having Prader–Willi Syndrome.[5,7,10] Furthermore, pre- and post-natal growth failure, prominent forehead, micrognathia, and muscular hypotonia during early infancy are often found in Silver–Russell Syndrome (SRS) (OMIM 180860).[11] To our knowledge; however, UPD(14)mat has been identified only in a single patient diagnosed as having SRS with no description of detailed phenotype.[12]

Here, we report on epimutations of the IG-DMR and the *MEG3*-DMR in two patients with SRS-compatible phenotype, and discuss on phenotypic overlap between SRS and TS.

PATIENTS AND METHODS

Patients

We studied 85 Japanese SRS patients in whom underlying genetic factors remained unknown from our previous study for 138 SRS patients[13] who satisfied the mandatory criteria and at least three of the five scoring system criteria proposed by Netchine *et al*[14] (for details of the criteria, see footnote of Table 1). In the previous study,[13] we identified *H19*-DMR hypomethylation (epimutation) in 43 patients (31.2%) and UPD(7)mat in nine patients (6.5%), and revealed a microdeletion at chromosome 17q24 in a single patient by analyzing copy number alterations for chromosome 11p15.5, 7p12.2, 12q14, and 17q24 that have been identified in rare SRS patients.[15–18]

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Table 1 Assessment of Silver–Russell Syndrome (SRS) clinical findings

	Case 1	*Case 2*			
	46,XY	*46,XX*	*No. 445 ...(male)*	*TS patients*	*SRS patients*[a]
Karyotype genetic cause	*Epimutation*	*Epimutation*	*UPD(14)mat*	*UPD(14)mat (n = 44)*	*Unknown (n = 85)*
SRS diagnosis criteria[b]					
Mandatory criteria for SRS					
BL and/or BW ≤ −2 SDS	+	+	+	28/35	85/85
Scoring system criteria for SRS					
Relative macrocephaly at birth[c]	+	+	...	11/21	16/45[d]
PH ≤ −2 SDS at 2 years	+ (−2.2 SD)	+ (−3.6 SD)	+	21/37	52/61[d]
Prominent forehead	+	+	...	17/21	41/53[d]
Body asymmetry	+	+	−	1/1[e]	19/59[d]
Feeding difficulties	−	−	+	20/25	25/51[d]
Other findings					
Gestational age (weeks)	41	37	...	38 (26 ~ 42) (*n* = 34)	38 (27 ~ 41) (*n* = 65)
BL cm (SDS)	46.5 (−2.1)	36.5 (−6.0)	...	ND[f]	(−2.9 ± 1.4) (*n* = 60)
BW kg (SDS)	2.2 (−2.7)	1.2 (−4.6)	... (−2.6)	ND[f]	(−2.7 ± 1.1) (*n* = 64)
BOFC cm (SDS)	32.5 (−0.7)	30.0 (−2.0)	...	ND[f]	(1.9 ± 1.1) (*n* = 48)
Present age (years:months)	9:6	9:2	17:9	7:10 (0:3 ~ 30:0) (*n* = 43)	4:3 (0:1 ~ 18:6) (*n* = 60)
PH cm (SDS)	120.4 (−2.3)	125.5 (−1.0)[g]	... (0.4 centile)	ND[f]	(−3.2 ± 1.5) (*n* = 61)
PW kg (SDS)	26.5 (−0.7)	22.3 (−1.2)	... (0.4 centile)	ND[f]	(−2.8 ± 1.3) (*n* = 59)
BMI (kg/m^2) (SDS)	18.3 (+1.0) SD)	14.2 (−1.1)	...	...	...
POFC cm (SDS)	51.5 (−0.9)	50.3 (−1.5)	...	ND[f]	(−1.8 ± 1.6) (*n* = 35)
Relative macrocephaly at present[h]	−	−	...	10/20	29/43
Triangular face	+	+	...	2/12	65/65
Ear anomalies	−	−	...	2/5	15/55
Irregular teeth	+	−	+	2/3	12/45
Clinodactyly	+	+	+	6/6	50/58
Brachydactyly	+	−	−	6/6	34/56
Single palmar crease	+	−	...	7/7	6/49
Muscular hypotonia	+	−	−	29/40	12/50
Speech delay	+	−	−	5/11	18/43
Remark		IVF-ET			
Reference	This study	This study	Poole *et al*[12]	See Supplementary Table S4	Fuke *et al*[13]

Abbreviations: BL, birth length; BMI, body mass index; BOFC, birth occipitofrontal circumference; BW, birth weight; IVF-ET, *in vitro* fertilization-embryo transfer; ND, not determined; PH, present height; POFC, present occipitofrontal circumference; PW, present weight; SDS, standard deviation score; SRS, Silver–Russell Syndrome; TS, Temple Syndrome; UPD(14)mat, maternal uniparental disomy 14.

[a]Japanese SRS patients who have neither epimutation at the *H19*-DMR nor UPD(7)mat.

[b]The diagnosis of SRS is made when a patient is positive for the mandatory criteria and at least three of the five scoring system criteria (Netchine *et al*[14])

[c]BL or BW (SDS)-BOFC (SDS) ≤ −1.5.

[d]Of the 85 patients, none have all the five scoring system criteria, 19 exhibit four of the five scoring system criteria, and 66 manifest three of the scoring system criteria.

[e]The presence of body asymmetry has been documented only in a single patient; while the presence or the absence of body asymmetry is not described, it is inferred that body asymmetry is absent in most, if not all, patients who have been examined for UPD(14)mat.

[f]Not determined because of lack of precise data in several studies, different growth assessment (SDS or centile) among studies, and different ethnicity.

[g]The height increase was obviously due to central precocious puberty.

[h]BL or BW (SDS)-BOFC (SDS) ≤ −1.5.

For UPD(14)mat and SRS patients, the denominators indicate the number of patients examined for the presence or absence of each feature, and the numerators represent the number of patients assessed to be positive for that feature.In cases 1 and 2 and the 85 SRS patients, birth and present length/height, weight, and occipitofrontal circumference were assessed by the gestational/postnatal age- and sex-matched Japanese reference data from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Science, Sports and Culture. BMI was evaluated by Japanese reference data.[29]

The 85 patients had a less-typical SRS phenotype (for details, see Fuke *et al*[13]). Indeed, of the 85 patients, none showed all of the five Netchine scoring system features, and 19 and 66 patients manifested four and three scoring system features, respectively. By contrast, of the 43 patients with *H19*-DMR epimutations, 10 patients were positive for all the five Netchine scoring system features, and 16 and 17 patients exhibited four and three scoring system features, respectively. This phenotypic difference was primarily due to the difference in the frequencies of relative macrocephaly at birth (35.6% *vs* 100%) and body asymmetry (32.2% *vs* 81.1%) between the two groups; the frequencies of the remaining three scoring system features were similar between the two groups. As our previous study included a large number of such patients with less-typical SRS phenotype, this would explain why the prevalence of *H19*-DMR epimutations was lower in our previous study than in Western European studies reported in the literature.[11,14,19] The phenotypes of the nine UPD(7)mat patients fell between those of the 85 idiopathic SRS patients and those of the 43 epimutation-positive patients, with the frequencies of relative macrocephaly at birth and body asymmetry being 77.8% and 33.3%, respectively. This appeared to be consistent with the prevalence of UPD(7)mat being similar between our previous study and Western European studies.[11,15,19–21]

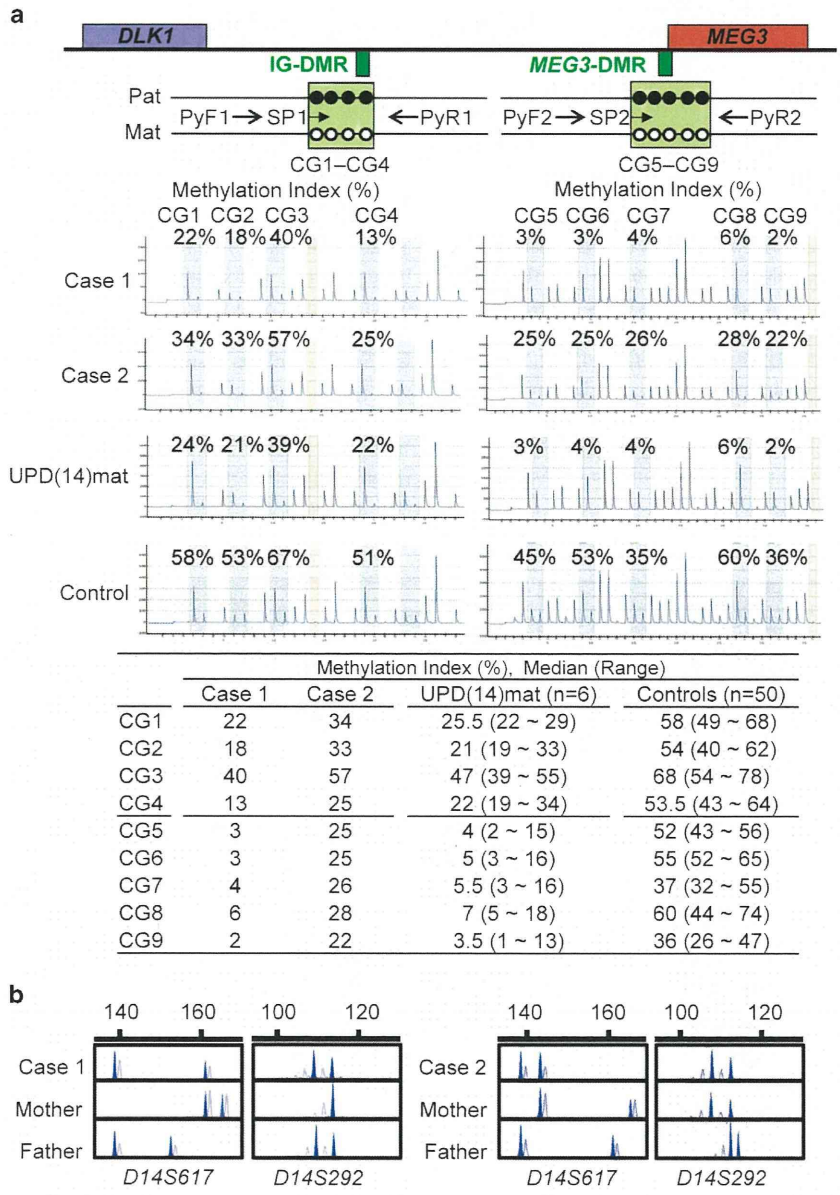
Ethical approval and samples

This study was approved by the Institute Review Board Committees of National Center for Child Health and Development and Hamamatsu University School of medicine, and performed using peripheral leukocyte samples after obtaining written informed consent.

Molecular studies

We first performed pyrosequencing analysis for four CpG dinucleotides (CG1–CG4) within the IG-DMR and five CpG dinucleotides (CG5–CG9) within the *MEG3*-DMR, using bisulfite-treated leukocyte genomic DNA samples (Figure 1). The procedure was as described in the manufacturer's instructions (Qiagen, Valencia, CA, USA). Subsequently, methylation indices (MIs, the ratio of methylated clones) were obtained using PyroMark Q24 (Qiagen). We also studied six UPD(14)mat patients for comparison and 50 control subjects to define the reference ranges of MIs.

When hypomethylation was identified, we performed microsatellite analysis for nine loci on chromosome 14, FISH analysis for the IG-DMR and the *MEG3*-DMR, and array comparative genomic hybridization for the 14q32.2 imprinted region using a custom-build oligo-microarray containing 12 600 probes (Agilent Technologies, Palo Alto, CA, USA).[22] We also performed pyrosequencing for the *H19*-DMR (ICR1) and the *PEG1/MEST*-DMR to re-confirm the absence of the known causes for SRS, and for the KvDMR (ICR2), the *SNRPN*-DMR, the *PLAGL1*-DMR, and the *GNAS* exon A/B-DMR to examine the occurrence of



	Methylation Index (%), Median (Range)			
	Case 1	Case 2	UPD(14)mat (n=6)	Controls (n=50)
CG1	22	34	25.5 (22 ~ 29)	58 (49 ~ 68)
CG2	18	33	21 (19 ~ 33)	54 (40 ~ 62)
CG3	40	57	47 (39 ~ 55)	68 (54 ~ 78)
CG4	13	25	22 (19 ~ 34)	53.5 (43 ~ 64)
CG5	3	25	4 (2 ~ 15)	52 (43 ~ 56)
CG6	3	25	5 (3 ~ 16)	55 (52 ~ 65)
CG7	4	26	5.5 (3 ~ 16)	37 (32 ~ 55)
CG8	6	28	7 (5 ~ 18)	60 (44 ~ 74)
CG9	2	22	3.5 (1 ~ 13)	36 (26 ~ 47)

Figure 1 Representative molecular findings. (**a**) Methylation analysis by pyrosequencing analysis. Top panel: schematic representation indicating of four CpG dinucleotides (CG1–CG4) within the IG-DMR and five CpG dinucleotides (CG5–CG9) within the *MEG3*-DMR. The cytosine residues at the CpG dinucleotides are usually methylated after paternal transmission (filled circles) and unmethylated after maternal transmission (open circles). A 164 bp segment encompassing CG1–CG4 and a 167 bp segment harboring CG5–CG9 were PCR amplified with primer sets (PyF1-PyR1 and PyF2-PyR2) hybridizing to both methylated and unmethylated clones, and sequence primers (SP1 and SP2) were hybridized to single-stranded PCR products. Middle panel: pyrosequencing data in cases 1 and 2, a UPD(14)mat patient, and a control subject. Bottom panel: summary of MIs. (**b**) Microsatellite analysis. The data are consistent with biparental origin of the chromosome 14 pairs. Unequal amplification of the heterozygous peaks in each individual is consistent with short products being more easily amplified than long products, and the patterns of heterozygous peak heights for *D14S292* are comparable between case 1 and the father and between case 2 and the mother, with no disproportionally increased heights of maternally derived peaks.

multiple methylation defects.[23] Primers utilized in this study are shown in Supplementary Table 1.

RESULTS

Molecular studies

Pyrosequencing identified hypomethylation of the IG-DMR and the *MEG3*-DMR in two of the 85 SRS patients (cases 1 and 2) (Figure 1). The MIs in case 1 were around the lower limit of the MIs in the six UPD(14)mat patients and much lower than the reference range in the 50 control subjects, whereas the MIs in case 2 were above the maximum MIs in the six UPD(14)mat patients, except for the MI of CG4, and below the reference range in the 50 controls, except for the MI of CG3. The MIs were obviously lower at the *MEG3*-DMR than at the IG-DMR in case 1 and the six UPD(14)mat patients, whereas the MIs were not so different between the IG-DMR and the *MEG3*-DMR in case 2 and the 50 control subjects.

In cases 1 and 2, microsatellite analysis showed biparental transmission of the homologs of chromosome 14, with similar patterns of peak heights for heterozygous alleles between cases and the parents (eg, comparable patterns of peak heights for the 108 bp and the 112 bp alleles of *D14S292* between case 1 and the father and between case 2 and the mother) (Figure 1 and Supplementary Table 2). FISH analysis delineated two copies of the IG-DMR and the *MEG3*-DMR, and array comparative genomic hybridization revealed no discernible copy number alteration in the 14q32.2 imprinted region (Supplementary Figure 1). Furthermore, the MIs for the six DMRs other than the IG-DMR and the *MEG3*-DMR were invariably within the normal range in cases 1 and 2 (Supplementary Table 3).

Clinical findings of cases 1 and 2

Both cases 1 and 2 showed severe prenatal growth failure, the mandatory criteria (ie, birth length and/or birth weight ≤ -2 SD), and four of the five scoring system criteria (ie, relative macrocephaly at birth, postnatal short stature (≤ -2 SD) at ≥ 2 year of age, prominent forehead during early childhood, and body asymmetry) for the diagnosis of SRS, whereas both of them lacked feeding difficulties (Table 1 and Figure 2). In addition, both cases 1 and 2 exhibited triangular face and clinodactyly, and case 1 manifested irregular teeth, brachydactyly, Single palmar crease, muscular hypotonia, and speech delay. Notably, relative macrocephaly with prominent forehead was no longer recognizable with age in both cases. Consistent with this, although the facial appearance was fairly characteristic of SRS in both cases in infancy to early childhood, it became less characteristic in both cases with age (Figure 2).

Both cases 1 and 2 also exhibited TS (UPD(14)mat) clinical features (Supplementary Table 4). In particular, several features characteristic of TS rather than SRS were observed, such as the body mass index above the mean at 9 years of age (though not assessed as obese), joint hypermobility, and small hands in case 1, and small hands and early onset of puberty in case 2.

Clinical survey also revealed that case 2 was born after *in vitro* fertilization-embryo transfer, whereas case 1 was born after natural conception. Furthermore, case 1 was treated with growth hormone for short stature from 6 to 8 years of age, and case 2 received growth hormone therapy for short stature since 5 years of age and gonadotropin-releasing hormone analog therapy for precocious puberty since 7 years of age.

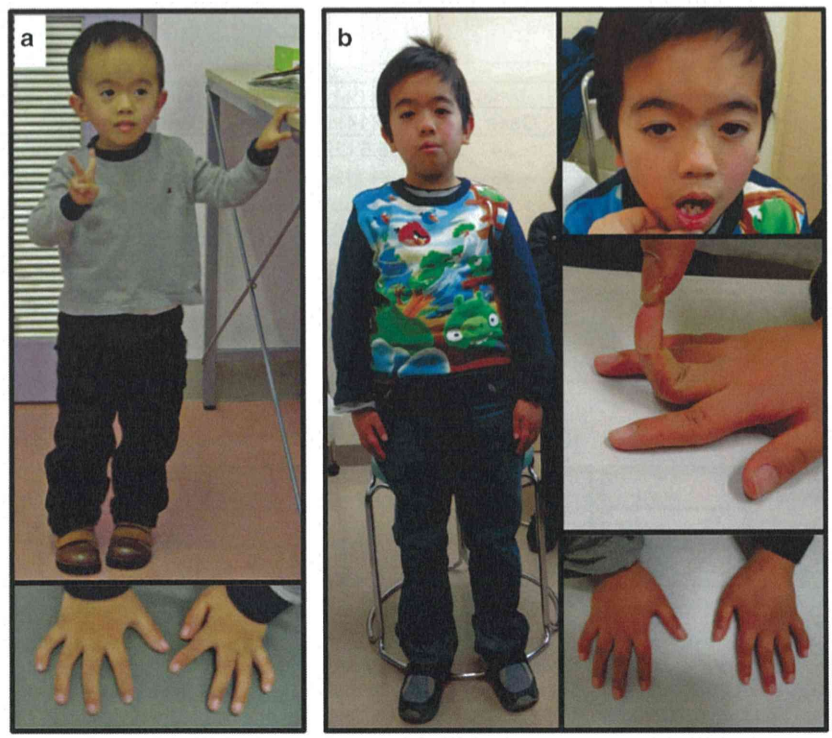


Figure 2 Photographs of case 1. (**a**) At 3 5/12 years of age. He exhibits triangular face with prominent forehead and micrognathia, and clinodactyly of the 5th fingers. (**b**) At 9 6/12 years of age. He exhibits slight central obesity, with the body mass index above the mean. Although this photo suggests mild scoliosis, this is primarily due to body asymmetry with asymmetric leg length. No scoliosis has been identified at the sitting position. He also manifests irregular teeth, joint hypermobility, and clinodactyly of the 5th fingers.

DISCUSSION

The present study showed that the IG-DMR and the *MEG3*-DMR were severely hypomethylated in case 1 with the MIs comparable to those of UPD(14)mat and moderately hypomethylated in case 2 with the MIs between those of UPD(14)mat patients and those of control subjects, in the absence of UPD(14)mat and microdeletion or copy number alteration involving the DMRs. Furthermore, although such hypomethylation patterns, especially the moderate hypomethylation in case 2, could be caused by post zygotic mosaicism with maternal full or distal 14q segmental isodisomy involving the imprinted region,[24] microsatellite analysis indicated no disproportionally increased height of the maternally inherited alleles, thereby arguing against the possible mosaicism. Taken together, the results imply the occurrence of epimutation (hypomethylation) of the IG-DMR and the *MEG3*-DMR in cases 1 and 2.

Cases 1 and 2 satisfied SRS diagnostic criteria proposed by Netchine *et al.*[14] In addition, UPD(14)mat has been identified in a single patient diagnosed as having SRS, although detailed clinical findings are unknown (No. 445 in Table 1).[12] Furthermore, phenotypic assessment of TS patients with UPD(14)mat reported in the literature reveals that such patients frequently exhibit clinical features utilized as the mandatory and the scoring system criteria for SRS (Table 1). Indeed, pre- and post-natal growth failure, prominent forehead, and feeding difficulties are shared in common by SRS and TS (Table 1 and Supplementary Table 4). In this regard, although the presence or the absence of body asymmetry is not described in most TS patients, it is unlikely that body asymmetry was not reported despite its presence (body asymmetry has been described in a single patient with UPD(14)mat and Prader–Willi Syndrome-like phenotype).[25] Thus, it is inferred that a considerable degree of phenotypic overlap exists between SRS and TS, except for body asymmetry that is apparently characteristic of SRS, and that epimutations of the IG-DMR and the *MEG3*-DMR were identified in cases 1 and 2 who exceptionally manifested body asymmetry.

Several matters should be pointed out in this study. First, the MIs were obviously lower at the *MEG3*-DMR than at the IG-DMR in case 1 and the six UPD(14)mat patients, whereas the MIs were not so different between the IG-DMR and the *MEG3*-DMR in case 2 and the 50 control subjects. As the IG-DMR and the *MEG3*-DMR function as the imprinting centers in the placenta and the body, respectively,[26] hypomethylation may be more strictly established in the *MEG3*-DMR of leukocytes in patients with UPD(14)mat and definitive epimutation. Second, multiple methylation defects was not detected in cases 1 and 2. Although the examined DMRs were rather limited, this may argue that isolated epimutation of the IG-DMR and the *MEG3*-DMR can lead to SRS phenotype. Third, relative macrocephaly with prominent forehead became clinically non-recognizable with age in cases 1 and 2. Thus, although clinical features of the two cases were compatible with SRS with no specific finding that serves to distinguish the two cases from other SRS patients in infancy to early childhood, they became less characteristic for SRS with age. Indeed, except for body asymmetry, their recent clinical features were more similar to those of patients with TS[4,8] or those of patients with short stature born small-for-date with no catch-up growth.[27] Such phenotypic change with age, in addition to TS-like clinical features such as recent body mass index gain in case 1 and early onset of puberty in case 2, might be characteristic of SRS patients with an aberrant chromosome 14 imprinted region. Fourth, case 2 was born after *in vitro* fertilization. As *in vitro* fertilization could be a risk factor for the occurrence of epimutation (hypomethylation),[28] *in vitro* fertilization may be related to the moderate degree of epimutation in case 2. Lastly, epimutation was identified only in two of the 85 SRS patients who were free from epimutation of the *H19*-DMR and UPD(7)mat. Poole *et al*[12] also have identified UPD(14)mat in one of 127 SRS patients, although clinical assessment remained fragmentary in 127 patients. Thus, UPD(14)mat and related genetic aberrations account for only a small fraction of SRS patients, and underlying factor(s) still remain to be clarified in many SRS patients. Nevertheless, analysis of the chromosome 14 imprinted region is worth attempting in SRS patients, especially in those with neither hypomethylation of the *H19*-DMR nor UPD(7)mat.

In summary, we identified epimutations affecting the IG-DMR and the *MEG3*-DMR in two patients with SRS-compatible phenotype. Further studies will permit to define the phenotypic spectrum of TS with aberrations of the chromosome 14 imprinted region.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes

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Human chromosome 14q32.2 carries a cluster of imprinted genes including paternally expressed genes (PEGs) such as *DLK1* and *RTL1* and maternally expressed genes (MEGs) such as *MEG3* (also known as *GTL2*), *RTL1as* (*RTL1* antisense) and *MEG8* (refs. 1,2), together with the intergenic differentially methylated region (IG-DMR) and the *MEG3*-DMR[3–5]. Consistent with this, paternal and maternal uniparental disomy for chromosome 14 (upd(14)pat and upd(14)mat) cause distinct phenotypes[6,7]. We studied eight individuals (cases 1–8) with a upd(14)pat-like phenotype and three individuals (cases 9–11) with a upd(14)mat-like phenotype in the absence of upd(14) and identified various deletions and epimutations affecting the imprinted region. The results, together with recent mouse data[4,8–10], imply that the IG-DMR has an important *cis*-acting regulatory function on the maternally inherited chromosome and that excessive *RTL1* expression and decreased *DLK1* and *RTL1* expression are relevant to upd(14)pat-like and upd(14)mat-like phenotypes, respectively.

Upd(14)pat results in a unique phenotype characterized by facial abnormality, a small, bell-shaped thorax, abdominal wall defects and polyhydramnios[6], and upd(14)mat leads to clinically discernible features such as pre- and postnatal growth failure and early onset of puberty[7]. We identified five individuals with a typical upd(14)pat phenotype (cases 1, 2 and 6–8) and three individuals with a relatively mild upd(14)pat-like phenotype (cases 3–5); we also identified three individuals with a upd(14)mat-like phenotype (cases 9–11), among whom case 11 had severely compromised adult height (**Table 1** and **Supplementary Tables 1–3** online). Cases 1–8 were identified because of the presence of a bell-shaped thorax in the neonatal period (**Supplementary Fig. 1** online), and cases 9–11 were ascertained through familial studies of cases 1–8. Thus, cases 1 and 2 and cases 9 and 10 were identified in the same family (family A), as were case 3 and case 11 (family B) (**Fig. 1**). The remaining cases, 4–8, were sporadic. All karyotypes were normal except for 46,XY,r(14)(p11q32.2) in case 5, and upd(14) was excluded in all cases by microsatellite analysis (**Supplementary Table 4** online).

We examined the 14q32.2 imprinted region (**Fig. 2**) using leukocyte genomic DNA and lymphocyte metaphase spreads of cases 2–11 (case 1 was deceased) and their family members who were willing to participate in this study. We also analyzed blood samples of control subjects and previously reported upd(14)pat and upd(14) mat cases[6,11].

We first determined the DMRs to be examined in this study (**Supplementary Fig. 2a** online). For the IG-DMR[4], *in silico* analysis followed by bisulfite sequencing revealed two DMRs, which we

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Table 1 Summary of clinical and molecular findings

	Cases 1[a] & 2	Case 3	Case 4	Case 5	Cases 6–8	Cases 9 & 10	Case 11
Upd(14)pat-like phenotype	+ (typical)	+ (mild)	+ (mild)	+ (mild)	+ (typical)		
Upd(14)mat-like phenotype						+	+ (severe)[b]
IG-DMR	Deleted	Deleted	Deleted	Deleted	Epimutated[c]	Deleted	Deleted
MEG3-DMR	Deleted	Deleted	Deleted	Deleted	Epimutated[c]	Deleted	Deleted
Deleted PEGs	*DLK1*	*DLK1*	*RTL1*	*DLK1*	None	*DLK1*	*DLK1*
		RTL1		*RTL1*			*RTL1*
				DIO3			
Deleted MEGs	*MEG3*	*MEG3*	*MEG3*	*MEG3*	None	*MEG3*	*MEG3*
		RTL1as	*RTL1as*	*RTL1as*			*RTL1as*
		MEG8	*MEG8*	*MEG8*			*MEG8*
Parental origin[d]	Maternal	Maternal	Maternal	Maternal	Maternal	Paternal	Paternal

Detailed clinical features of cases 1–8 and upd(14)pat cases are described in **Supplementary Table 1**, and those of cases 9–11 and upd(14)mat cases are described in **Supplementary Table 2**. Phenotypic assessment is summarized in **Supplementary Table 3**. Chest roentgenograms of cases 1–8 are shown in **Supplementary Fig. 1**.
[a]Case 1, though not studied, presumably has the same deletion as case 2. [b]Adult height is severely compromised in case 11. [c]Hypermethylation of the normally hypomethylated allele of maternal origin (**Fig. 3**). [d]Parental origin of chromosomes with deletions or epimutations. In cases 7, 10 and 11, although parental genotyping data are not informative or available, methylation and FISH analyses indicate hypermethylation of the normally hypomethylated allele of maternal origin in case 7 and loss of the normally hypermethylated allele of paternal origin in cases 10 and 11 (**Fig. 3** and **Fig. 4**).

designated CG4 and CG6. For the *MEG3*-DMR, we confirmed the previously reported DMR[5] (hereafter designated CG7) through bisulfite sequencing and PCR amplification with methylated and unmethylated allele-specific primers.

We then carried out methylation analysis, which showed that the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) were severely hypermethylated in cases 2–8, to an extent comparable to that found in the upd(14)pat case, whereas they were grossly hypomethylated in cases 9–11, to a degree similar to that identified in the upd(14)mat case (**Fig. 3** and **Supplementary Fig. 2b**). Notably, we confirmed hypermethylation of normally hypomethylated CG4 clones of maternal origin by informative SNP typing data in cases 6 and 8. We carried out FISH analysis with two long and accurate (LA)-PCR products covering the IG-DMR and the *MEG3*-DMR, and we found familial heterozygous microdeletions in cases from families A and B and a *de novo* heterozygous microdeletion in case 4 (**Fig. 4**). This deletion was also detected in case 5, the individual with the r(14) chromosome, but it was absent in cases 6–8.

Subsequently, we carried out genotyping analysis for 200 loci, showing lack of common alleles for multiple loci between cases 2 and 9 and between cases 9 and 10 in family A, between case 3 and case 11 in family B, between case 4 and the mother, and between case 5 and the mother (**Supplementary Table 4**). We carried out sequencing analyses for LA-PCR products obtained with primers flanking the deleted loci and identified a 108,768-bp deletion involving *DLK1* and *MEG3* in cases 2, 9 and 10 of family A (case 1 presumably had the same deletion), a 411,354-bp deletion involving *WDR25*, *BEGAIN*, *DLK1*, *MEG3*, *RTL1*, *RTL1as* and *MEG8* in cases 3 and 11 of family B, and a 474,550-bp deletion involving *MEG3*, *RTL1*, *RTL1as* and *MEG8* in case 4; in case 5, we carried out FISH analyses with nine BAC probes covering the imprinted region and identified a ~6.5-Mb deletion involving the whole imprinted region (**Fig. 2** and **Supplementary Fig. 3** online). In cases 6–8, we identified neither tiny deletion nor sequence variation around the *DLK1-MEG3* region, including the DMRs and the putative CTCF binding sites[12], by extensive analyses (**Supplementary Fig. 3**), and we found normal methylation patterns for DMRs at the *MEST* promoter[13], the *IGF2-H19* domain[14] and the *SNRPN* promoter[15]. Thus, we determined that cases 6–8 have epimutations affecting the 14q32.2 imprinted region. Although we attempted to examine the expression dosage of the imprinted genes on 14q32.2, expression was absent or extremely faint in leukocytes. The above molecular data from leukocytes are summarized in **Table 1**.

We further examined placental samples, because virtually all the imprinted genes studied to date are expressed in the placenta[16,17]. Using expression analyses combined with SNP genotypings, we confirmed monoallelic paternal expression of *DLK1* and maternal expression of *MEG3* in normal fresh placentas (**Supplementary Fig. 2c**). RT-PCR analyses showed positive PEGs expression and negative MEGs expression in formalin-treated and paraffin-embedded placental samples of cases 2 and 8 and the upd(14)pat case, with *RTL1* expression being obviously elevated in the three placentas compared to a similarly treated control placenta (**Fig. 5a**). Histological examinations showed proliferation of dilated and congested chorionic villi in the three placentas (**Fig. 5b**). Furthermore, CG4 was hypermethylated in the placentas of cases 2 and 8 and the upd(14)pat case and was delineated as the DMR in the control placenta, whereas CG7 was rather hypomethylated in the four placentas and did not show the DMR-compatible methylation patterns (**Supplementary Fig. 2c**).

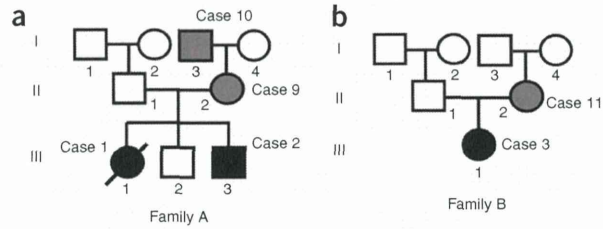

Figure 1 The pedigrees of two families. (**a**) Family A. Case 1 (III-1) and case 2 (III-3) show typical upd(14)pat phenotype (black), whereas case 9 (II-2) and case 10 (I-3) manifest upd(14)mat-like phenotype with mild to moderate short stature (gray). The remaining five family members have normal phenotype. (**b**) Family B. Case 3 (III-1) shows relatively mild upd(14)pat-like phenotype (black), and case 11 has upd(14)mat-like phenotype with marked short stature (gray). The remaining five family members, including the maternal grandparents, have normal phenotype (stature, ± 0 s.d. in the maternal grandfather and –0.8 s.d. in the maternal grandmother). The maternal grandparents refused to take part in molecular studies.