located in this partner-factor interaction region, which is important for the cell-specific actions of SOX2. Missense mutations in this region may lead to phenotypic variability in ocular malformation and/or HH due not only to residual SOX2 activity, but also the interaction with tissue-specific partner factors.

We used a next-generation sequencing strategy to analyze 122 genes associated with congenital endocrine disorders. This approach is new in HH; it has never been reported to our knowledge. The genetic etiologies of HH are quite heterogeneous, and recent investigations have revealed that HH is not strictly a monogenic mendelian disease as previously thought; instead, it is emerging as a digenic and potentially oligogenic disease [17, 18]. When multiple genes need to be analyzed for mutations simultaneously, targeted sequence analysis of interesting genomic regions is an attractive approach.

In summary, this study expands the range of known molecular defects in SOX2, and extends our understanding of the phenotypic features and developmental disease course associated with mutations in *SOX2*.

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Disclosure Statement

The authors have nothing to disclose.

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ORIGINAL ARTICLE

Clinical and molecular studies in four patients with *SRY*-positive 46,XX testicular disorders of sex development: implications for variable sex development and genomic rearrangements

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We report four patients with *SRY*-positive 46,XX testicular disorders of sex development (46,XX-TDSD) (cases 1–4). Case 1 exhibited underdeveloped external genitalia with hypospadias, case 2 manifested micropenis and cases 3 and 4 showed normal external genitalia. The Xp;Yp translocations occurred between the X- and the Y-differential regions in case 1, between *PRKX* and inverted *PRKY* in case 2 and between the X-chromosomal short arm pseudoautosomal region and the Y-differential regions in cases 3 and 4. The distance of the Yp breakpoint from *SRY* was ~0.75 Mb in case 1, ~6.5 Mb in case 2, ~2.3 Mb in case 3 and ~72 kb in case 4. The Xp;Yp translocation occurred within an 87-bp homologous segment of *PRKX* and *PRKY* in case 2, and between non-homologous regions with addition of an 18-bp sequence of unknown origin in case 4. X-inactivation analysis revealed random inactivation in cases 1–4. The results argue against the notion that undermasculinization in 46,XX-TDSD is prone to occur when translocated Yp materials are small (<100 kb of the Y-differential region), and imply that the Xp;Yp translocations result from several mechanisms including non-allelic homologous recombination and non-homologous end joining.
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INTRODUCTION

SRY-positive 46,XX testicular disorders of sex development (46,XX-TDSD) (previously known as XX maleness) is a relatively rare condition resulting from unbalanced Xp;Yp translocations during paternal meiosis.[1,2] This condition is associated with normal male external genitalia in 80–90% of patients and undermasculinized external genitalia in the remaining 10–20% of patients.[1,2] Since undermasculinized genitalia are prone to occur when translocated Yp materials are small (<100 kb of the Y-differential region),[3] it has been suggested that spreading of X-inactivation into *SRY* or change in the *SRY* position relative to chromosomal environments (position effect) has compromised *SRY* expression, leading to undermasculinization.[1–3] By contrast, when large Yp materials (several Mb of the Y-differential region) are translocated onto Xp, the presence of abundant Yp materials would have protected *SRY* from being subjected to X-inactivation or position effect, permitting normal male development.[1–3] However, some exceptional patients exhibit normal male genitalia under translocations of small Yp materials,[4,5] and other exceptional patients show undermasculinized external genitalia under translocations of relatively large Yp materials.[3,6,7] Thus, a straightforward explanation is difficult for the variable sex development in 46,XX-TDSD.

The Xp;Yp translocations can be caused by non-allelic homologous recombination (NAHR).[8] In particular, previous studies have revealed the recurrent NAHR events between X-Y homologous sequences around *PRKX* and *PRKY*, especially at the 'hot spot A' on the 5′ sequence and the 'hot spot B' on the C-terminal coding sequence, in a subgroup of males with a common ~3.5 Mb paracentric Yp inversion mediated by Y-specific long inverted repeats.[9] Since *PRKX* and *PRKY* are aligned in the same direction during paternal meiosis in such Yp inversion-positive males, this would have permitted the occurrence of NAHR around *PRKX* and *PRKY*.[10] However, the positions of Xp and the Yp breakpoints are variable among patients, and the genomic basis for Xp;Yp translocations remains to be clarified in most patients, especially in those born to Yp inversion-negative males.

Here, we report on molecular studies in four patients with *SRY*-positive 46,XX-TDSD, and discuss on variable sex development and genomic rearrangements in *SRY*-positive TDSD.

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Table 1 Clinical and molecular findings in cases 1–4 with 46,XX-TDSD

	Case 1	*Case 2*	*Case 3*	*Case 4*
Present age (years:months)	2:5	2:6	23:0	3:11
Reason for karyotyping	Genital abnormalities	Branchial arch syndrome[a]	Premature delivery[b]	Advanced pregnant age (37 years)[c]
External genitalia	Microphallus[d] Cryptorchidism (L) Penoscrotal hypospadias	Micropenis[e]	Normal male	Normal male
Penile length (cm)	2.3 (−3.0 SD) at 3 months	2.5 (−2.8 SD) at 3 months	NE	NE
Internal genitalia	Uterus-like structure	NE	No Müllerian structure[f]	NE
Testis size (ml)	1–2 (B) (1–2)	2 (B) (1–2)	Small testes	2 (B) (1–2)
Basal serum hormones				
Examined age (years:months)	0:3	2:3	18:6	0:8
Testosterone ($ng\,ml^{-1}$)	4.69 (0.03–3.5)	<0.05 (0.03–0.13)	2.1 (1.3–8.7)	NE
LH ($mIU\,ml^{-1}$)	2.6 (0.2–3.6)	<0.3 (<0.3)	17.8 (0.8–5.7)	NE
FSH ($mIU\,ml^{-1}$)	6.7 (0.5–4.4)	0.62 (0.4–2.1)	32.2 (2.0–8.3)	NE
Xp breakpoint	Proximal to *NLGN4X*	Within *PRKX*	Within PAR1	Within PAR1
Yp breakpoint	Proximal to *ZFY*	Within inverted *PRKY*	Within *PCDH11Y*	Within *RPS4Y1*
Distance from *SRY*	~0.75 Mb	~6.5 Mb	~2.3 Mb	~72 kb
X-inactivation ratio[g]	79%:21% (X^P:X^M)	84%:16% (X^P:X^M)	56%:44%	47%:53%

Abbreviations: B, bilateral; FSH, follicle stimulating hormone; L, left; LH, luteinizing hormone; NE, not examined; PAR1, the Xp/Yp pseudoautosomal region; SDS, standard deviation score; X^M, maternally derived X chromosome; X^P, paternally derived X chromosome; 46,XX-TDSD, 46,XX testicular disorders of sex development.
The values in brackets represent age-matched Japanese reference data.
[a]Left hemifacial microsomia, microtia with narrow auditory canal, mild microphthalmia and micrognathia.
[b]Chromosome analysis was performed for intrauterine growth retardation, and case 3 was informed of the 46,XX karyotype at 18 years of age.
[c]Karyotype analysis was examined for amniocytes at 16 weeks of gestation and for umbilical cord lymphocytes at birth.
[d]Increased to 3.2 cm after testosterone enanthate therapy (25 mg per dose, 4 × injections).
[e]Increased to 3.5 cm after testosterone enanthate therapy (25 mg per dose, 2 × injections).
[f]No Müllerian structures were identified on pelvic magnetic resonance imaging; laparoscopy was not performed.
[g]The ratio of inactive X chromosomes.

MATERIALS AND METHODS

Patients and ethical approval

We studied hitherto undescribed four patients with 46,XX-TDSD (cases 1–4) in whom fluorescence *in situ* hybridization delineated *SRY* on the tip of one of the two X chromosomes and direct sequencing showed normal *SRY* sequence. This study was approved by the Institutional Review Board Committee of Hamamatsu University School of Medicine, and performed after obtaining written informed consent from the parents of the child subjects (cases 1, 2 and 4) and from the adult subject (case 3).

Samples and primers

Molecular studies were performed using peripheral leukocytes of cases 1–4 and the parents of cases 1 and 2. The primers utilized in this study are shown in Supplementary Table 1.

Array comparative genomic hybridization analysis

Oligonucleotide-based array comparative genomic hybridization (CGH) was performed, using a custom-build array containing 522 888 probes for an ~67-Mb distal Xp region and 87 464 probes for an ~15-Mb distal Yp region, as well as ~10 000 reference probes for other chromosomal regions. In case 2 with branchial arch syndrome phenotype, we also performed array CGH using the Agilent G4447A Sure Print G3 Human CGH 1 × 1M Oligo Microarray kit (Agilent Technologies, Santa Clara, CA, USA) containing 1 million catalog probes for the whole genome. Obtained copy number variants/polymorphisms were screened with the Agilent Genomic Workbench software (Agilent Technologies) using the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home). The procedure was as described in the manufacturer's instructions.

Determination of the Xp;Yp translocation fusion point

We performed long PCR amplification using multiple primers that were designed to hybridize to single copy sequences around the breakpoints indicated by array CGH. Single copy sequences were identified by Repeatmasker (http://www.repeatmasker.org). When long PCR products were obtained, they were sequentially sequenced, and obtained sequences were compared with the X and the Y reference sequences (GRCh37/hg19, http://genome.ucsc.edu/). The homology between the Xp and the Yp sequences harboring the breakpoints was examined by Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

X-inactivation analysis

X-inactivation pattern was analyzed by the methylation assay for *AR* (exon 1), as reported previously.[11] The parental origin of the two X chromosomes was determined by genotyping for CAG repeat length polymorphism within the amplified segments before *Hpa*II digestion.

RESULTS

Clinical findings

Clinical findings are summarized in Table 1. Case 1 had undermasculinized external genitalia and was raised as a male, after thorough consultation with the parents. He received testosterone therapy for microphallus (a small penis associated with other external genital abnormalities such as hypospadias)[12] during infancy and surgical operation for the correction of hypospadias and orchidopexy at 16 months of age. Macroscopic examination at the time of operation showed apparently normal testes and a uterine remnant; the uterine remnant was not removed, because there were no clinical problems such as infection. Testicular biopsy for microscopic examination was refused by the parents. Case 2 had male external genitalia with micropenis (a small penis without other external genital abnormalities)[12] that required testosterone therapy. Cases 3 and 4 exhibited normal male external genitalia, while adult case 3 had apparently small testes. Case 2 also had branchial arch syndrome phenotype. The reason for karyotyping was variable in cases 1–4. Endocrine studies revealed apparently normal findings with sufficiently elevated basal serum testosterone value during mini-puberty in case 1, age-appropriated data in case 2 and relative hypergonadotropic hypogonadism in adult case 3.

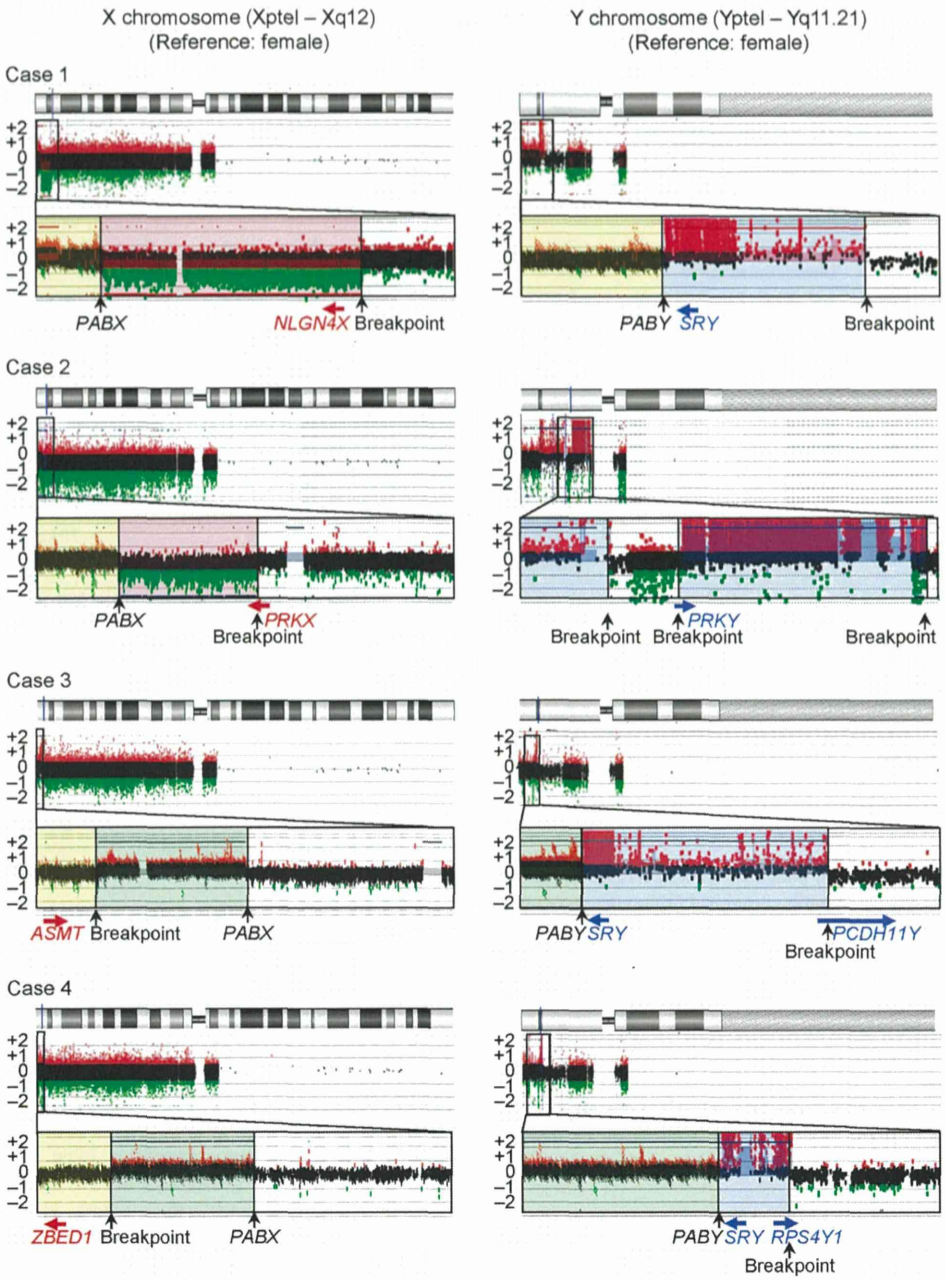

Figure 1 Representative results of array CGH analysis. The black, the red, and the green dots denote signals indicative of the normal, the increased (log2 signal ratio > +0.5), and the decreased (log2 signal ratio < −0.8) copy numbers, respectively. The log2 signal ratios of +0.5 and −1.0 indicate the presence of three copies and a single copy of the corresponding regions, respectively. The segments highlighted with red, blue, yellow, and green rectangles represent the deleted X-differential region, the translocated Y-differential region, the PAR1 with the normal copy numbers, and the PAR1 with increased copy numbers, respectively.

Array CGH analysis

The results are shown in Figure 1 and summarized in Table 1. Case 1 had two copies of the Xp/Yp short arm pseudoautosomal region (PAR1), deletion of an ~3.8-Mb X-differential region with the breakpoint proximal to *NLGN4X*, and translocation of an ~0.75-Mb Y-differential region with the breakpoint proximal to *ZFY*. Case 2 had two copies of the PAR1, deletion of an ~1.0-Mb X-differential region with the breakpoint on *PRKX*, and complex translocations of an ~3.8-Mb and an ~2.7-Mb Y-differential regions with the middle breakpoint on *PRKY* and the centromeric and the telomeric breakpoints around the long inverted repeats for the common ~3.5 Mb paracentric Yp inversion. Cases 3 and 4 had an increased copy number of a proximal part of the PAR1 and a normal copy number of a distal part of the PAR1, and possessed the entire Xp-differential region and a very distal part of the Yp-differential region, with the different breakpoints. In case 2, no other copy number variation, which was not registered in the Database of Genomic Variants, was detected by the whole genome catalog array.

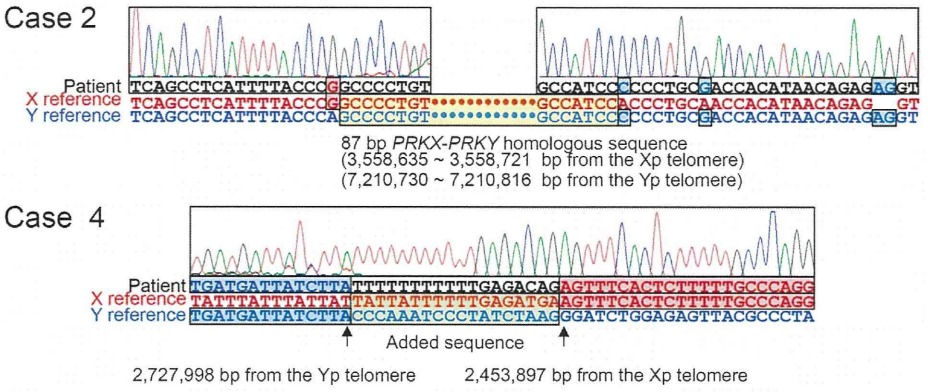

Figure 2 Precise translocation fusion points. The translocation fusion point in case 2 is located within an X-Y homologous 87-bp segment (indicated by a yellow rectangle) between *PRKX*- and *PRKY*-specific nucleotides that are highlighted in red and blue, respectively; although only a single *PRKX*-specific nucleotide is shown in this figure, multiple *PRKX*-specific nucleotides are identified in the more centromeric portion. The translocation fusion point in case 4 resides between the X- and the Y-specific sequences that are highlighted in red and blue, respectively, and is accompanied by an 18 nucleotide sequence of unknown origin (indicated by a green rectangle).

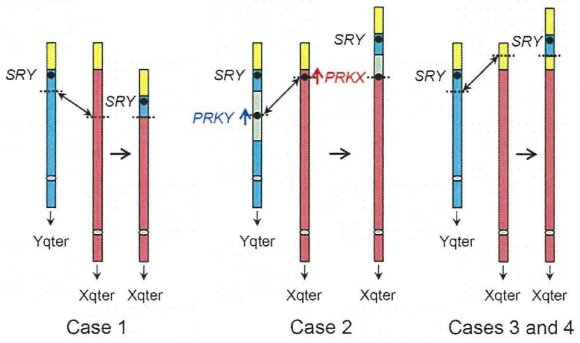

Figure 3 Schematic representation of the aberrant translocations. The yellow, the orange, the blue, and the green segments denote the PAR1, the X-differential region, the Y-differential region, and the Y-differential inverted region, respectively.

Determination of the Xp;Yp translocation fusion point

Long PCR products harboring the fusion points were obtained in cases 2 and 4, and the precise translocation fusion points were determined by direct sequencing (Figure 2); in case 2, long PCR amplification was carried out on the assumption of paternal Yp inversion. The fusion point in case 2 resided within an 87-bp homologous segment at intron 4 of *PRKX* and *PRKY*. The fusion point in case 4 was associated with 18 nucleotides of unknown origin that was inserted between the 'A' nucleotide at the position 2 453 897 bp from the Xptel and the 'A' nucleotide at the position 2 727 998 bp from the Yptel. There was no sequence homology between the Xp and the Yp breakpoint regions in case 4.

In cases 1 and 3, long PCR products were not obtained, probably because of the presence of repetitive sequences around the Yp breakpoint in case 1 and the Xp breakpoint in case 3; in addition, the Xp breakpoint in case 3 resided on a copy number variant region.

X-inactivation analysis

The two X chromosomes were randomly inactivated in cases 1–4, although the paternally derived X chromosome harboring *SRY* was somewhat preferentially inactivated in cases 1 and 2 (Table 1; Supplementary Figure 1).

DISCUSSION

The present study indicates that the Xp;Yp translocations occurred between the X- and the Y-differential regions in case 1, between *PRKX* and inverted *PRKY* in case 2, and between the X-chromosomal PAR1 and the Y-differential regions in cases 3 and 4 (Figure 3). This suggests that the Xp;Yp translocations can take place between various Xp and Yp regions, and provides further support for a critical role of a common paracentric Yp inversion in the occurrence of the *PRKX*/*PRKY*-mediated Xp;Yp translocation.[10] Notably, the combination of the relatively large Xp deletion and the relatively large Yp translocation observed in case 1 has not been described previously, although the translocations between homologous sequences encompassing *PRKX* and *PRKY* observed in case 2 and between the Xp-PAR1 and the very distal part of the Yp-differential region observed in cases 3 and 4 have been reported previously.[3,10,13] Furthermore, the results of case 2 represent that the *PRKX*/*PRKY*-mediated Xp;Yp translocation can occur outside the 'hot spot A' and the 'hot spot B'.

Clinical and molecular findings in this study argue against the notion that undermasculinization is prone to occur when translocated Yp materials are small. The translocated Yp material was relatively large in case 1 with undermasculinized external genitalia and quite small in case 4 with normal male genitalia. In addition, while the *SRY*-positive paternally derived X chromosome was somewhat preferentially inactivated in case 1, previous studies have indicated no correlation between the degree of masculinization and X-inactivation patterns.[3,14] Indeed, if X-inactivation is spread into *SRY*, then this would cause ovotesticular disorders of sex development and undermasculinized genitalia under random X-inactivation. One may argue that the location of Xp breakpoints have an important role in sex development, because *SRY* might escape or undergo X-inactivation when the Yp materials are attached to normally active or inactive X regions, respectively. However, the Xp breakpoints of cases 1–4 resided in the regions that primarily, if not completely, escape X-inactivation.[15]

Thus, the underlying primary factors for undermasculinization may be variable among patients with *SRY*-positive 46,XX-TDSD, including spreading of X-inactivation and position effect. In this regard, case 1 had macroscopically normal testes and sufficiently elevated basal serum testosterone during mini-puberty, although defective testis formation and resultant testosterone hyposecretion

are postulated for undermasculinization in *SRY*-positive 46,XX-TDSD.[16] It might be possible, however, that *SRY* expression was transiently dysregulated during fetal life because of a position effect caused by an altered three-dimensional chromatin structure that is more or less specific to the Xp;Yp translocation in case 1.[17] Such possible transient dysregulation of *SRY* expression might also be relevant to micropenis in case 2. Furthermore, since postnatal endocrine status can grossly be normal in patients with mutations or deletions of testis formation genes such as *DMRT1* and *NR5A1* (*SF1*),[18,19] the apparently normal endocrine data would not necessarily exclude the macroscopically undetectable defective testis structure in case 1. Similarly, while case 4 exhibited apparently normal external genitalia, this would not necessarily exclude the possibility of impaired testis formation and resultant testosterone hyposecretion of a certain degree. Since the translocations of small Yp materials onto the Xp-PAR1 are associated with both apparently normal external genitalia and undermasculinized external genitalia,[3,13] diverse genital phenotype may be regarded as a continuous spectrum.

The precise Xp;Yp translocation fusion points were determined in cases 2 and 4. Although the *PRKX*/*PRKY*-mediated translocation in case 2 is ascribed to NAHR, the translocation in case 4 occurred between non-homologous regions and was associated with an 18-bp sequence of unknown origin. Such genomic structure at the fusion point is characteristic of non-homologous end joining that can lead to various genomic rearrangements including translocations.[8] It is likely, therefore, that Xp;Yp translocations are caused by several mechanisms for genomic rearrangements, although Xp;Yp translocations resulting from replication-based mechanisms such as fork stalling and template switching and microhomology-mediated break-induced replication remain to be identified at present.[8]

In addition to 46,XX-TDSD, case 2 had branchial arch syndrome. Thus, case 2 would have a branchial arch syndrome-related genetic aberration that could not be identified by the whole genome array CGH. In this regard, an X-linked recessive form of branchial arch syndrome has been reported,[20] although there is no other report describing branchial arch syndrome in patients with 46,XX-TDSD. Thus, it might be possible that the gene for branchial arch syndrome was lost from the paternally derived X chromosome, and that the homologous gene on the maternally derived X chromosome was mutated or selectively inactivated in tissues expressing that gene.

In summary, the results exemplify the complexity of underlying factors involved in sex development in 46,XX-TDSD, and imply that the Xp;Yp translocations are caused by several mechanisms including NAHR and non-homologous end joining. For sex development in 46,XX-TDSD, while the overall data currently available would still support the notion that undermasculinization in 46,XX-TDSD is prone to occur when translocated Yp materials are small, several exceptional patients have been identified including cases 1 and 4 in this study. Further studies will permit to clarify the underlying factors for variable sex development and genomic basis of Xp;Yp translocations in 46,XX-TDSD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Clinical and molecular studies in four patients with *SRY*-positive 46,XX testicular disorders of sex development: implications for variable sex development and genomic rearrangements

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We report four patients with *SRY*-positive 46,XX testicular disorders of sex development (46,XX-TDSD) (cases 1–4). Case 1 exhibited underdeveloped external genitalia with hypospadias, case 2 manifested micropenis and cases 3 and 4 showed normal external genitalia. The Xp;Yp translocations occurred between the X- and the Y-differential regions in case 1, between *PRKX* and inverted *PRKY* in case 2 and between the X-chromosomal short arm pseudoautosomal region and the Y-differential regions in cases 3 and 4. The distance of the Yp breakpoint from *SRY* was ~0.75 Mb in case 1, ~6.5 Mb in case 2, ~2.3 Mb in case 3 and ~72 kb in case 4. The Xp;Yp translocation occurred within an 87-bp homologous segment of *PRKX* and *PRKY* in case 2, and between non-homologous regions with addition of an 18-bp sequence of unknown origin in case 4. X-inactivation analysis revealed random inactivation in cases 1–4. The results argue against the notion that undermasculinization in 46,XX-TDSD is prone to occur when translocated Yp materials are small (<100 kb of the Y-differential region), and imply that the Xp;Yp translocations result from several mechanisms including non-allelic homologous recombination and non-homologous end joining.
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INTRODUCTION

SRY-positive 46,XX testicular disorders of sex development (46,XX-TDSD) (previously known as XX maleness) is a relatively rare condition resulting from unbalanced Xp;Yp translocations during paternal meiosis.[1,2] This condition is associated with normal male external genitalia in 80–90% of patients and undermasculinized external genitalia in the remaining 10–20% of patients.[1,2] Since undermasculinized genitalia are prone to occur when translocated Yp materials are small (<100 kb of the Y-differential region),[3] it has been suggested that spreading of X-inactivation into *SRY* or change in the *SRY* position relative to chromosomal environments (position effect) has compromised *SRY* expression, leading to undermasculinization.[1–3] By contrast, when large Yp materials (several Mb of the Y-differential region) are translocated onto Xp, the presence of abundant Yp materials would have protected *SRY* from being subjected to X-inactivation or position effect, permitting normal male development.[1–3] However, some exceptional patients exhibit normal male genitalia under translocations of small Yp materials,[4,5] and other exceptional patients show undermasculinized external genitalia under translocations of relatively large Yp materials.[3,6,7] Thus, a straightforward explanation is difficult for the variable sex development in 46,XX-TDSD.

The Xp;Yp translocations can be caused by non-allelic homologous recombination (NAHR).[8] In particular, previous studies have revealed the recurrent NAHR events between X-Y homologous sequences around *PRKX* and *PRKY*, especially at the 'hot spot A' on the 5′ sequence and the 'hot spot B' on the C-terminal coding sequence, in a subgroup of males with a common ~3.5 Mb paracentric Yp inversion mediated by Y-specific long inverted repeats.[9] Since *PRKX* and *PRKY* are aligned in the same direction during paternal meiosis in such Yp inversion-positive males, this would have permitted the occurrence of NAHR around *PRKX* and *PRKY*.[10] However, the positions of Xp and the Yp breakpoints are variable among patients, and the genomic basis for Xp;Yp translocations remains to be clarified in most patients, especially in those born to Yp inversion-negative males.

Here, we report on molecular studies in four patients with *SRY*-positive 46,XX-TDSD, and discuss on variable sex development and genomic rearrangements in *SRY*-positive TDSD.

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Table 1 Clinical and molecular findings in cases 1–4 with 46,XX-TDSD

	Case 1	*Case 2*	*Case 3*	*Case 4*
Present age (years:months)	2:5	2:6	23:0	3:11
Reason for karyotyping	Genital abnormalities	Branchial arch syndrome[a]	Premature delivery[b]	Advanced pregnant age (37 years)[c]
External genitalia	Microphallus[d] *Cryptorchidism* (L) Penoscrotal hypospadias	Micropenis[e]	Normal male	Normal male
Penile length (cm)	2.3 (−3.0 SD) at 3 months	2.5 (−2.8 SD) at 3 months	NE	NE
Internal genitalia	Uterus-like structure	NE	No Müllerian structure[f]	NE
Testis size (ml)	1–2 (B) (1–2)	2 (B) (1–2)	Small testes	2 (B) (1–2)
Basal serum hormones				
Examined age (years:months)	0:3	2:3	18:6	0:8
Testosterone (ng ml^{-1})	4.69 (0.03–3.5)	<0.05 (0.03–0.13)	2.1 (1.3–8.7)	NE
LH (mIU ml^{-1})	2.6 (0.2–3.6)	<0.3 (<0.3)	17.8 (0.8–5.7)	NE
FSH (mIU ml^{-1})	6.7 (0.5–4.4)	0.62 (0.4–2.1)	32.2 (2.0–8.3)	NE
Xp breakpoint	Proximal to *NLGN4X*	Within *PRKX*	Within PAR1	Within PAR1
Yp breakpoint	Proximal to *ZFY*	Within inverted *PRKY*	Within *PCDH11Y*	Within *RPS4Y1*
Distance from *SRY*	~0.75 Mb	~6.5 Mb	~2.3 Mb	~72 kb
X-inactivation ratio[g]	79%:21% (X^P:X^M)	84%:16% (X^P:X^M)	56%:44%	47%:53%

Abbreviations: B, bilateral; FSH, follicle stimulating hormone; L, left; LH, luteinizing hormone; NE, not examined; PAR1, the Xp/Yp pseudoautosomal region; SDS, standard deviation score; X^M, maternally derived X chromosome; X^P, paternally derived X chromosome; 46,XX-TDSD, 46,XX testicular disorders of sex development.
The values in brackets represent age-matched Japanese reference data.
[a]Left hemifacial microsomia, microtia with narrow auditory canal, mild microphthalmia and micrognathia.
[b]Chromosome analysis was performed for intrauterine growth retardation, and case 3 was informed of the 46,XX karyotype at 18 years of age.
[c]Karyotype analysis was examined for amniocytes at 16 weeks of gestation and for umbilical cord lymphocytes at birth.
[d]Increased to 3.2 cm after testosterone enanthate therapy (25 mg per dose, 4× injections).
[e]Increased to 3.5 cm after testosterone enanthate therapy (25 mg per dose, 2× injections).
[f]No Müllerian structures were identified on pelvic magnetic resonance imaging; laparoscopy was not performed.
[g]The ratio of inactive X chromosomes.

MATERIALS AND METHODS

Patients and ethical approval

We studied hitherto undescribed four patients with 46,XX-TDSD (cases 1–4) in whom fluorescence *in situ* hybridization delineated *SRY* on the tip of one of the two X chromosomes and direct sequencing showed normal *SRY* sequence. This study was approved by the Institutional Review Board Committee of Hamamatsu University School of Medicine, and performed after obtaining written informed consent from the parents of the child subjects (cases 1, 2 and 4) and from the adult subject (case 3).

Samples and primers

Molecular studies were performed using peripheral leukocytes of cases 1–4 and the parents of cases 1 and 2. The primers utilized in this study are shown in Supplementary Table 1.

Array comparative genomic hybridization analysis

Oligonucleotide-based array comparative genomic hybridization (CGH) was performed, using a custom-build array containing 522 888 probes for an ~67-Mb distal Xp region and 87 464 probes for an ~15-Mb distal Yp region, as well as ~10 000 reference probes for other chromosomal regions. In case 2 with branchial arch syndrome phenotype, we also performed array CGH using the Agilent G4447A Sure Print G3 Human CGH 1 × 1M Oligo Microarray kit (Agilent Technologies, Santa Clara, CA, USA) containing 1 million catalog probes for the whole genome. Obtained copy number variants/polymorphisms were screened with the Agilent Genomic Workbench software (Agilent Technologies) using the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home). The procedure was as described in the manufacturer's instructions.

Determination of the Xp;Yp translocation fusion point

We performed long PCR amplification using multiple primers that were designed to hybridize to single copy sequences around the breakpoints indicated by array CGH. Single copy sequences were identified by Repeatmasker (http://www.repeatmasker.org). When long PCR products were obtained, they were sequentially sequenced, and obtained sequences were compared with the X and the Y reference sequences (GRCh37/hg19, http://genome.ucsc.edu/). The homology between the Xp and the Yp sequences harboring the breakpoints was examined by Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

X-inactivation analysis

X-inactivation pattern was analyzed by the methylation assay for *AR* (exon 1), as reported previously.[11] The parental origin of the two X chromosomes was determined by genotyping for CAG repeat length polymorphism within the amplified segments before *HpaII* digestion.

RESULTS

Clinical findings

Clinical findings are summarized in Table 1. Case 1 had undermasculinized external genitalia and was raised as a male, after thorough consultation with the parents. He received testosterone therapy for microphallus (a small penis associated with other external genital abnormalities such as hypospadias)[12] during infancy and surgical operation for the correction of hypospadias and orchidopexy at 16 months of age. Macroscopic examination at the time of operation showed apparently normal testes and a uterine remnant; the uterine remnant was not removed, because there were no clinical problems such as infection. Testicular biopsy for microscopic examination was refused by the parents. Case 2 had male external genitalia with micropenis (a small penis without other external genital abnormalities)[12] that required testosterone therapy. Cases 3 and 4 exhibited normal male external genitalia, while adult case 3 had apparently small testes. Case 2 also had branchial arch syndrome phenotype. The reason for karyotyping was variable in cases 1–4. Endocrine studies revealed apparently normal findings with sufficiently elevated basal serum testosterone value during mini-puberty in case 1, age-appropriated data in case 2 and relative hypergonadotropic hypogonadism in adult case 3.

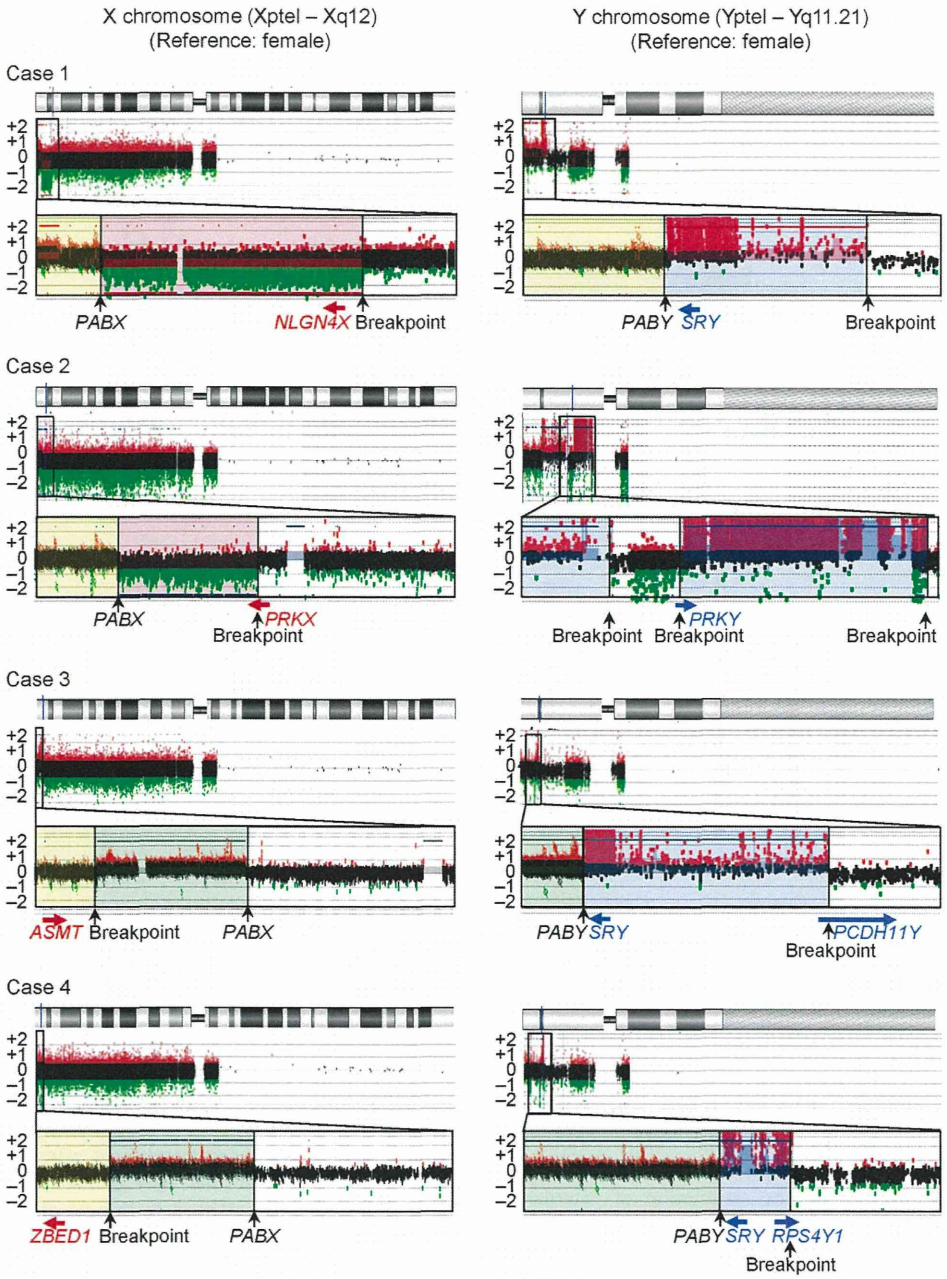

Figure 1 Representative results of array CGH analysis. The black, the red, and the green dots denote signals indicative of the normal, the increased (log2 signal ratio > +0.5), and the decreased (log2 signal ratio < −0.8) copy numbers, respectively. The log2 signal ratios of +0.5 and −1.0 indicate the presence of three copies and a single copy of the corresponding regions, respectively. The segments highlighted with red, blue, yellow, and green rectangles represent the deleted X-differential region, the translocated Y-differential region, the PAR1 with the normal copy numbers, and the PAR1 with increased copy numbers, respectively.

Array CGH analysis

The results are shown in Figure 1 and summarized in Table 1. Case 1 had two copies of the Xp/Yp short arm pseudoautosomal region (PAR1), deletion of an ~3.8-Mb X-differential region with the breakpoint proximal to *NLGN4X*, and translocation of an ~0.75-Mb Y-differential region with the breakpoint proximal to *ZFY*. Case 2 had two copies of the PAR1, deletion of an ~1.0-Mb X-differential region with the breakpoint on *PRKX*, and complex translocations of an ~3.8-Mb and an ~2.7-Mb Y-differential regions with the middle breakpoint on *PRKY* and the centromeric and the telomeric breakpoints around the long inverted repeats for the common ~3.5 Mb paracentric Yp inversion. Cases 3 and 4 had an increased copy number of a proximal part of the PAR1 and a normal copy number of a distal part of the PAR1, and possessed the entire Xp-differential region and a very distal part of the Yp-differential region, with the different breakpoints. In case 2, no other copy number variation, which was not registered in the Database of Genomic Variants, was detected by the whole genome catalog array.

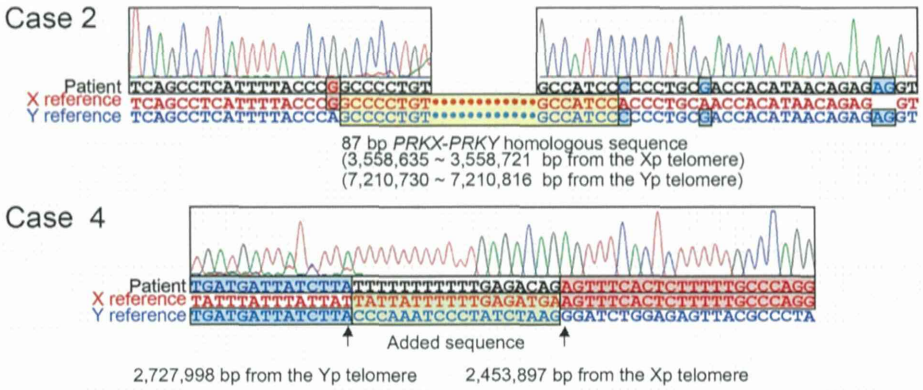

Figure 2 Precise translocation fusion points. The translocation fusion point in case 2 is located within an X-Y homologous 87-bp segment (indicated by a yellow rectangle) between *PRKX*- and *PRKY*-specific nucleotides that are highlighted in red and blue, respectively; although only a single *PRKX*-specific nucleotide is shown in this figure, multiple *PRKX*-specific nucleotides are identified in the more centromeric portion. The translocation fusion point in case 4 resides between the X- and the Y-specific sequences that are highlighted in red and blue, respectively, and is accompanied by an 18 nucleotide sequence of unknown origin (indicated by a green rectangle).

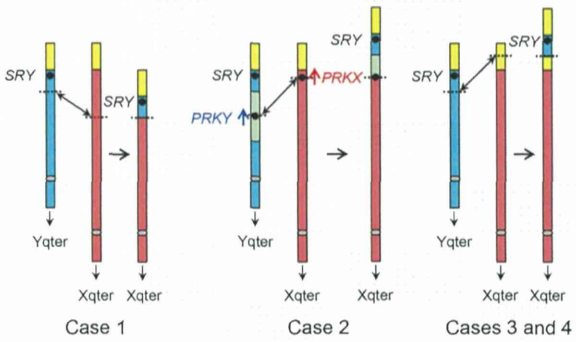

Figure 3 Schematic representation of the aberrant translocations. The yellow, the orange, the blue, and the green segments denote the PAR1, the X-differential region, the Y-differential region, and the Y-differential inverted region, respectively.

Determination of the Xp;Yp translocation fusion point

Long PCR products harboring the fusion points were obtained in cases 2 and 4, and the precise translocation fusion points were determined by direct sequencing (Figure 2); in case 2, long PCR amplification was carried out on the assumption of paternal Yp inversion. The fusion point in case 2 resided within an 87-bp homologous segment at intron 4 of *PRKX* and *PRKY*. The fusion point in case 4 was associated with 18 nucleotides of unknown origin that was inserted between the 'A' nucleotide at the position 2 453 897 bp from the Xptel and the 'A' nucleotide at the position 2 727 998 bp from the Yptel. There was no sequence homology between the Xp and the Yp breakpoint regions in case 4.

In cases 1 and 3, long PCR products were not obtained, probably because of the presence of repetitive sequences around the Yp breakpoint in case 1 and the Xp breakpoint in case 3; in addition, the Xp breakpoint in case 3 resided on a copy number variant region.

X-inactivation analysis

The two X chromosomes were randomly inactivated in cases 1–4, although the paternally derived X chromosome harboring *SRY* was somewhat preferentially inactivated in cases 1 and 2 (Table 1; Supplementary Figure 1).

DISCUSSION

The present study indicates that the Xp;Yp translocations occurred between the X- and the Y-differential regions in case 1, between *PRKX* and inverted *PRKY* in case 2, and between the X-chromosomal PAR1 and the Y-differential regions in cases 3 and 4 (Figure 3). This suggests that the Xp;Yp translocations can take place between various Xp and Yp regions, and provides further support for a critical role of a common paracentric Yp inversion in the occurrence of the *PRKX*/*PRKY*-mediated Xp;Yp translocation.[10] Notably, the combination of the relatively large Xp deletion and the relatively large Yp translocation observed in case 1 has not been described previously, although the translocations between homologous sequences encompassing *PRKX* and *PRKY* observed in case 2 and between the Xp-PAR1 and the very distal part of the Yp-differential region observed in cases 3 and 4 have been reported previously.[3,10,13] Furthermore, the results of case 2 represent that the *PRKX*/*PRKY*-mediated Xp;Yp translocation can occur outside the 'hot spot A' and the 'hot spot B'.

Clinical and molecular findings in this study argue against the notion that undermasculinization is prone to occur when translocated Yp materials are small. The translocated Yp material was relatively large in case 1 with undermasculinized external genitalia and quite small in case 4 with normal male genitalia. In addition, while the *SRY*-positive paternally derived X chromosome was somewhat preferentially inactivated in case 1, previous studies have indicated no correlation between the degree of masculinization and X-inactivation patterns.[3,14] Indeed, if X-inactivation is spread into *SRY*, then this would cause ovotesticular disorders of sex development and undermasculinized genitalia under random X-inactivation. One may argue that the location of Xp breakpoints have an important role in sex development, because *SRY* might escape or undergo X-inactivation when the Yp materials are attached to normally active or inactive X regions, respectively. However, the Xp breakpoints of cases 1–4 resided in the regions that primarily, if not completely, escape X-inactivation.[15]

Thus, the underlying primary factors for undermasculinization may be variable among patients with *SRY*-positive 46,XX-TDSD, including spreading of X-inactivation and position effect. In this regard, case 1 had macroscopically normal testes and sufficiently elevated basal serum testosterone during mini-puberty, although defective testis formation and resultant testosterone hyposecretion

are postulated for undermasculinization in *SRY*-positive 46,XX-TDSD.[16] It might be possible, however, that *SRY* expression was transiently dysregulated during fetal life because of a position effect caused by an altered three-dimensional chromatin structure that is more or less specific to the Xp;Yp translocation in case 1.[17] Such possible transient dysregulation of *SRY* expression might also be relevant to micropenis in case 2. Furthermore, since postnatal endocrine status can grossly be normal in patients with mutations or deletions of testis formation genes such as *DMRT1* and *NR5A1* (*SF1*),[18,19] the apparently normal endocrine data would not necessarily exclude the macroscopically undetectable defective testis structure in case 1. Similarly, while case 4 exhibited apparently normal external genitalia, this would not necessarily exclude the possibility of impaired testis formation and resultant testosterone hyposecretion of a certain degree. Since the translocations of small Yp materials onto the Xp-PAR1 are associated with both apparently normal external genitalia and undermasculinized external genitalia,[3,13] diverse genital phenotype may be regarded as a continuous spectrum.

The precise Xp;Yp translocation fusion points were determined in cases 2 and 4. Although the *PRKX*/*PRKY*-mediated translocation in case 2 is ascribed to NAHR, the translocation in case 4 occurred between non-homologous regions and was associated with an 18-bp sequence of unknown origin. Such genomic structure at the fusion point is characteristic of non-homologous end joining that can lead to various genomic rearrangements including translocations.[8] It is likely, therefore, that Xp;Yp translocations are caused by several mechanisms for genomic rearrangements, although Xp;Yp translocations resulting from replication-based mechanisms such as fork stalling and template switching and microhomology-mediated break-induced replication remain to be identified at present.[8]

In addition to 46,XX-TDSD, case 2 had branchial arch syndrome. Thus, case 2 would have a branchial arch syndrome-related genetic aberration that could not be identified by the whole genome array CGH. In this regard, an X-linked recessive form of branchial arch syndrome has been reported,[20] although there is no other report describing branchial arch syndrome in patients with 46,XX-TDSD. Thus, it might be possible that the gene for branchial arch syndrome was lost from the paternally derived X chromosome, and that the homologous gene on the maternally derived X chromosome was mutated or selectively inactivated in tissues expressing that gene.

In summary, the results exemplify the complexity of underlying factors involved in sex development in 46,XX-TDSD, and imply that the Xp;Yp translocations are caused by several mechanisms including NAHR and non-homologous end joining. For sex development in 46,XX-TDSD, while the overall data currently available would still support the notion that undermasculinization in 46,XX-TDSD is prone to occur when translocated Yp materials are small, several exceptional patients have been identified including cases 1 and 4 in this study. Further studies will permit to clarify the underlying factors for variable sex development and genomic basis of Xp;Yp translocations in 46,XX-TDSD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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

Karyotype genetic cause	*Case 1 46,XY Epimutation*	*Case 2 46,XX Epimutation*	*No. 445 ...(male) UPD(14)mat*	*TS patients UPD(14)mat (n = 44)*	*SRS patients[a] 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]

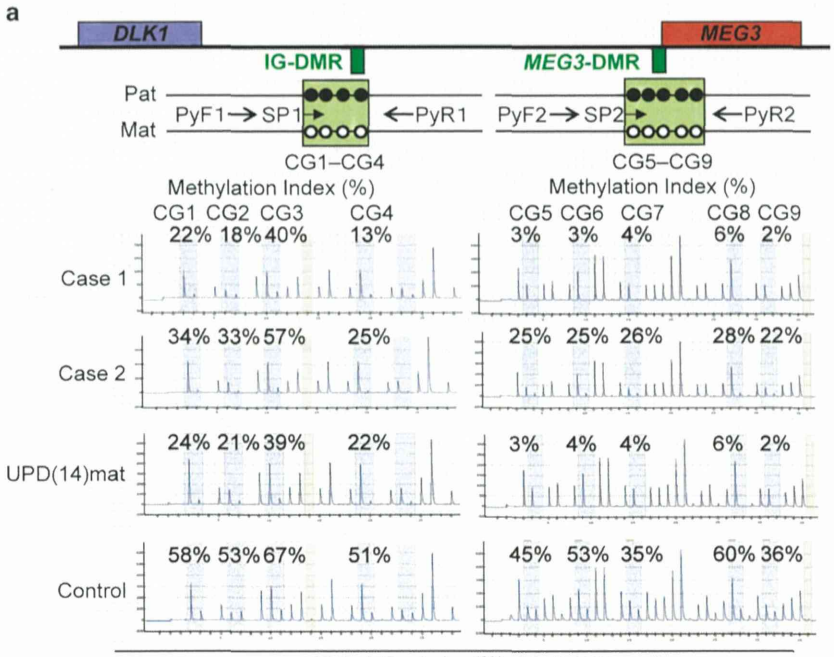
Ethical approval and samples

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)

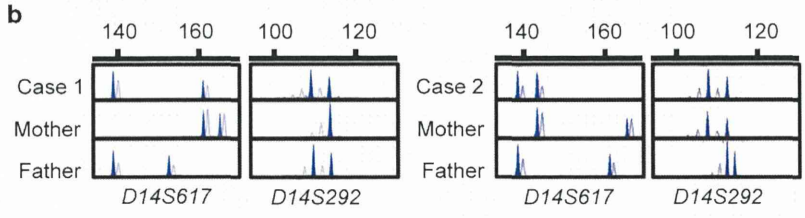


Figure 1 Representative molecular findings. (**a**) Methylation analysis by pyrosequencing analysis. Top panel: schematic representation indicating of four CpG dinucleotides (CG1–CG4) within the IG-DMR and five CpG dinucleotides (CG5–CG9) within the *MEG3*-DMR. The cytosine residues at the CpG dinucleotides 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.

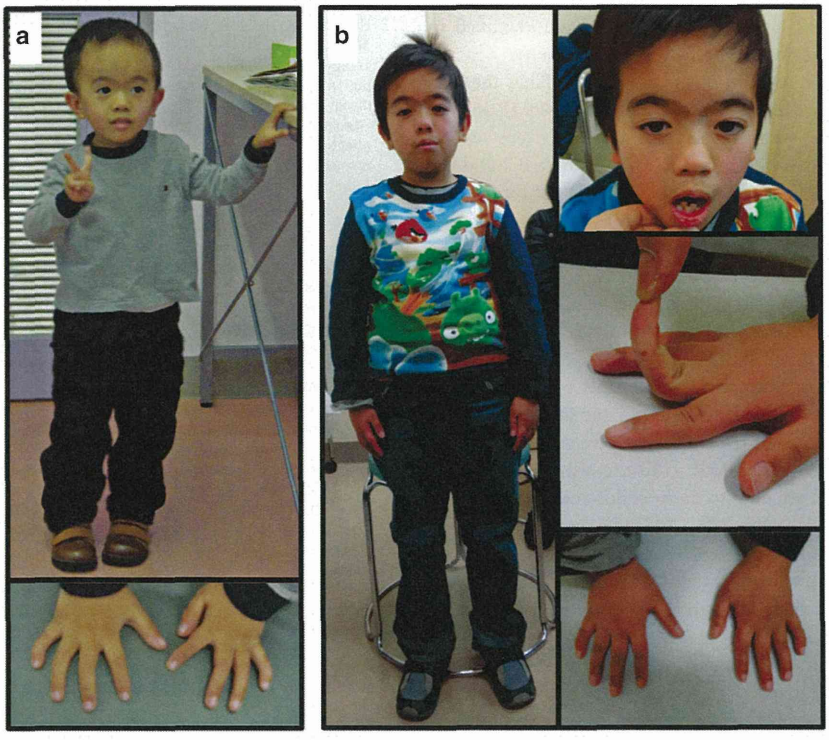


Figure 2 Photographs of case 1. (**a**) At 3 5/12 years of age. He exhibits triangular face with prominent forehead and micrognathia, and clinodactyly of the 5th fingers. (**b**) At 9 6/12 years of age. He exhibits slight central obesity, with the body mass index above the mean. Although this photo suggests 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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