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SHORT COMMUNICATION

Monozygotic female twins discordant for Silver–Russell syndrome and hypomethylation of the *H19*-DMR

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Abstract Silver–Russell syndrome (SRS) is characterized by growth failure and dysmorphic features, and is frequently caused by hypomethylation of the paternally derived *H19*-DMR (epimutation). We observed 5 8/12-year-old female twins discordant for SRS. One twin exhibited SRS-compatible features, such as pre- and postnatal growth failure, relative macrocephaly, triangular face, left hemihypotrophy, and bilateral fifth finger clinodactyly, whereas the other twin showed apparently normal phenotype. Microsatellite analysis for 26 loci on multiple chromosomes showed monozygosity. Methylation analysis for the *H19*-DMR indicated epimutation in roughly half of cells in the affected twin and normal patterns in the unaffected twin and the parents. X-inactivation analysis revealed random X-inactivation with a nearly identical pattern between the twins. The discordant methylation pattern of the *H19*-DMR may primarily be due to a failure to maintain the DNA methyltransferase-1-dependent methylation imprint around the pre-implantation S phase, because such failure would result in the production of two different cell clones, one with normally methylated DMR and the other with demethylated DMR, leading to the separation of cells with different characters and resultant twinning.

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Introduction

Silver–Russell syndrome (SRS; MIN 180860) is a developmental disorder characterized by pre- and postnatal growth failure, relative macrocephaly, triangular face, hemihypotrophy, and fifth-finger clinodactyly (Hitchins et al. 2001). Recent studies have shown that hypomethylation (epimutation) of the paternally derived differentially methylated region in the upstream of *H19* (*H19*-DMR) on chromosome 11p15 accounts for 30–65% of SRS patients (Gicquel et al. 2005; Bliek et al. 2006; Eggermann et al. 2006; Netchine et al. 2007). In this regard, it is known that a common enhancer is shared by the paternally expressed gene *IGF2* (insulin-like growth factor 2) and the maternally expressed gene *H19* (Leighton et al. 1995b), and that the enhancer exerts its effects on *IGF2* or *H19* depending on the methylation status of the *H19*-DMR (Leighton et al. 1995a; Thorvaldsen et al. 1998). This alternative enhancer effect is mediated by an insulator protein CTCF that binds to the unmethylated *H19*-DMR of maternal origin, but not to the methylated *H19*-DMR of paternal origin. Indeed, seven CTCF binding sites have been identified within the *H19*-DMR (Bell and Felsenfeld 2000; Hark et al. 2000). Thus, it has been postulated that the hypomethylation of the paternally derived *H19*-DMR results in maternalization of the *IGF2–H19* imprinted domain, leading to the development of SRS because of reduced *IGF2* expression (Gicquel et al. 2005). Although the exaggerated *H19* expression might also exert some clinical effect, this possibility is unlikely because the primary function of *H19* in

the bodies appears to be tumor suppression (Hao et al. 1993; Juan et al. 2000). This finding, together with the identification of maternal uniparental disomy for chromosome 7 [upd(7)mat] in 7–10% of SRS patients (Hitchins et al. 2001), implies that SRS primarily represents an epigenetic disorder.

Notably, five monozygotic twin pairs discordant for SRS have been reported (Nyhan and Sakati 1977; Samn et al. 1990; Bailey et al. 1995; Sagot et al. 1996; Gicquel et al. 2005). In addition, the *H19*-DMR analysis has been performed in a single female twin pair, identifying a discordant methylation pattern (Gicquel et al. 2005). Here, we report monozygotic twins discordant for SRS and hypomethylation of the *H19*-DMR, and discuss underlying factors for such discordance.

Materials and methods

Case report

This Japanese female patient and her twin sister were conceived naturally to an unrelated and healthy 52-year-old father and 37-year-old mother. They were found to be monochorionic diamniotic twins by ultrasonographic studies at the first trimester. Because of the advanced maternal age, amniocentesis was carried out at 15 weeks of gestation, showing a 46,XX karyotype in both of the twins. The patient exhibited growth retardation since 20 weeks of gestation, while the twin sister grew well. Since the patient frequently showed decelerated fetal heart rate at 28 weeks of gestation, the twins were delivered by an emergent cesarean section.

At birth, the patient was 25.0 cm in length, 494 g in weight, and 24.0 cm in occipitofrontal head circumference (OFC), whereas the twin sister was 32.0 cm in length, 734 g in weight, and 24.5 cm in OFC (no reference growth data in neonates born at 28 weeks). Both twins were admitted to the neonatal intensive care unit and received respiratory and nutrition management for long terms (10 months for the patient and 5 months for the sister). The placenta of 280 g was shared by the twin, but there was no discernible interconnecting vascular anastomosis. At 2 years of age, the patient was diagnosed as having SRS on the basis of a constellation of clinical features that met the diagnostic criteria for SRS (Hitchins et al. 2001), such as pre- and postnatal growth failure, triangular face, relative macrocephaly, left hemihypotrophy, and bilateral fifth finger clinodactyly. Her psychomotor development appeared to be age-appropriate. Endocrine studies for short stature were normal, as were radiological studies. By contrast, the twin sister showed catch-up growth and manifested no SRS-like features. At 5 8/12 years of age, the patient measured 91.2 cm (−4.5 SD), weighed 12.8 kg (−2.1 SD), and had an OFC of 48.3 cm (−1.4 SD), whereas the twin sister measured 102.0 cm (−1.9 SD), weighed 13.6 kg (−1.9 SD), and had an OFC of 49.1 cm (−0.9 SD).

Microsatellite analysis

This study was approved by the Institutional Review Board Committee at the National Center for Child health and Development, and written informed consent was obtained from the parents. A total of 26 loci on multiple chromosomes were genotyped, using leukocyte genomic DNA. In brief, a segment encompassing each locus was PCR-amplified with a fluorescently labeled forward primer and an unlabeled reverse primer, and was determined for the physical size on an ABI PRISM 310 autosequencer using GeneScan software (Applied Biosystems, Foster City, CA).

Methylation analysis

Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing were performed for two regions within the *H19*-DMR, i.e., a 317-bp region distal to the CTCF binding sites (DMR-A) (Vu et al. 2000) and a 435-bp region encompassing the CTCF binding site 6 (DMR-B), using leukocyte genomic DNA (Fig. 1a). The DMR-A and DMR-B were PCR-amplified with primers that hybridize to both methylated and unmethylated alleles because of absent CpG dinucleotides within the primer sequences.

For COBRA, the PCR products were digested with methylated allele-specific restriction enzymes (*Bsa*BI and *Mwo*I for the DMR-A; *Afl*III and *Hpy*188I for the DMR-B) (Fig. 1a). Subsequently, the methylation index was obtained using peak heights of digested and undigested fragments on the 2100 Bioanalyzer (Agilent, Santa Clara, CA). To define the normal range for the methylation indices, 40 control subjects were similarly studied.

For bisulfite sequencing, the PCR products were subcloned with TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and multiple clones were subjected to direct sequencing on the CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). The SNPs within the DMR-A (*rs2251375*) and the DMR-B (*rs11564736*, *rs10732516*, *rs34610866*, *rs2071094*, *rs35678657*, and *rs2107425*) were also genotyped, to identify the parental origin of each clone.

X-inactivation analysis

X-inactivation pattern was examined by the previously reported methods (Muroya et al. 1999). In brief, leukocyte genomic DNA was PCR-amplified with a

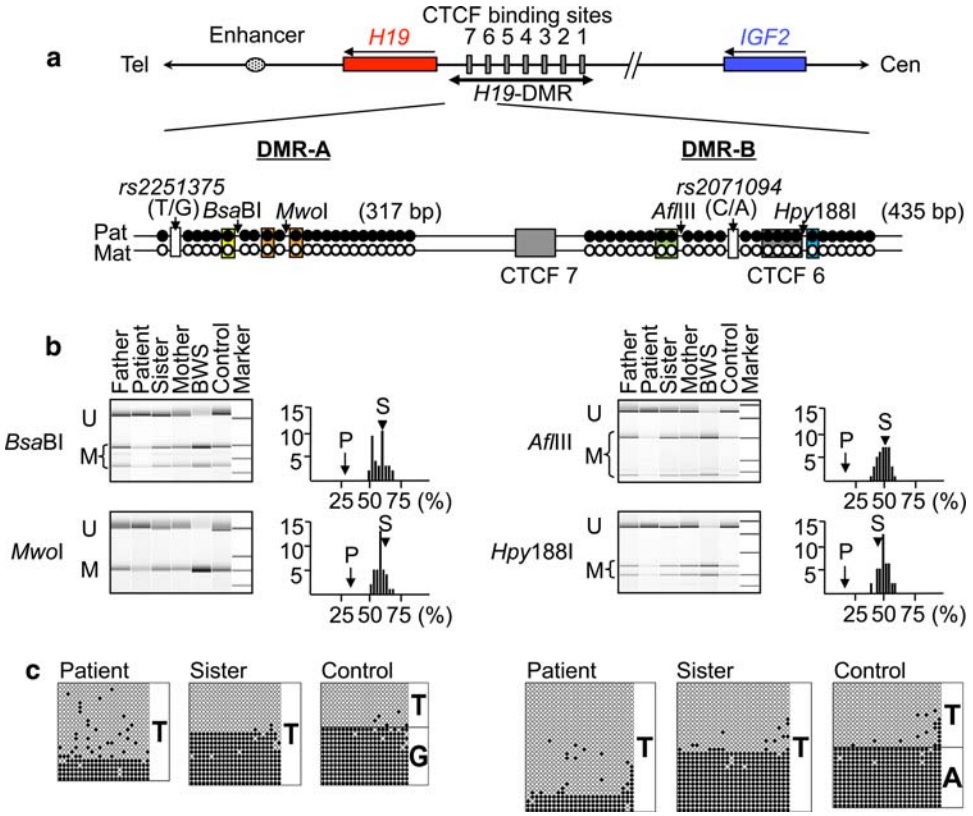

Fig. 1 Methylation analysis of the *H19*-DMR. **a** The regional physical map of the *IGF2-H19* imprinted domain and the location and the structure of the DMR-A and the DMR-B examined in this study. The *H19*-DMR resides in the upstream of *H19* and contains seven CTCF binding sites (shown in *gray boxes 1–7*). A common enhancer (shown with a *stippled ellipse*) is shared by the paternally expressed gene *IGF2* (shown in *blue*) and the maternally expressed gene *H19* (shown in *red*), and the enhancer exerts its effects on *IGF2* when the *H19*-DMR is methylated after paternal transmission (*filled circles*) and on *H19* when the *H19*-DMR is unmethylated after maternal transmission (*open circles*). The DMR-A (317 bp) resides distal to the CTCF binding sites and harbors 23 CpG dinucleotides, methylated allele-specific *Bsa*BI and *Mwo*I restriction sites, and the T/G SNP (*rs2251375*) (depicted with a *white box*). After bisulfite treatment, this region is digested with *Bsa*BI when the cytosine at the 6th CpG dinucleotide (indicated with a *yellow rectangle*) is methylated and with *Mwo*I when the two cytosines at the 9th and the 11th CpG dinucleotides (indicated with *two orange rectangles*) are methylated. The primer sequences used were: 5′-AACCCCTTCC-TACCACCATC-3′ and 5′-GGGTTTGGGAGAGTTTGTGA-3′. The DMR-B (435 bp) encompasses the CTCF binding site 6 (shown with a *gray box*) and contains 26 CpG dinucleotides, methylated allele-specific *Afl*III and *Hpy*188I restriction sites, and the C/A SNP (*rs2071094*) (depicted with a *white box*). After bisulfite treatment, this region is digested with *Afl*III when the cytosines at the eighth and the ninth CpG dinucleotides (indicated with *green rectangles*) are methylated and with *Hpy*188I when the cytosine at the 20th CpG dinucleotide (indicated with a *blue rectangle*) is methylated. The primer sequences used were: 5′-TGGGAGGAGATATTAGGGGA-ATA-3′ and 5′-TCCCAAACCATAACACTAAAACC-3′. **b** The results of the COBRA for the DMR-A (*left*) and the DMR-B (*right*). The actual electrophoresis images indicate that, for all the *Bsa*BI and the *Mwo*I sites in the DMR-A and the *Afl*III and the *Hpy*188I sites in the DMR-B, both unmethylated clone-specific bands (*U*) and methylated clone-specific bands (*M*) are found in the father, the sister, and the mother as well as in a control subject, whereas *U* is predominant in the patient and *M* is predominant in the Beckwith–Wiedemann syndrome (*BWS*) patient with upd(11p15)pat. As compared with the methylation indices of the normal control subjects depicted as histograms (the *horizontal axis* the methylation index with an interval of 2.5%; the *vertical axis* the subject number), the methylation indices of the patient (*P*) (shown with *arrows*) are below the normal range, and those of the twin sister (*S*) (shown with *arrowheads*) remain within the normal range. **c** Bisulfite sequencing results of the DMR-A (*left*) and the DMR-B (*right*) in the patient, the sister, and a control subject. *Each line* indicates a single clone, and each *circle* denotes a CpG dinucleotide; *filled* and *open circles* represent methylated and unmethylated cytosines, respectively. The T/G SNP (*rs2251375*) typing data within the DMR-A are indicated, as are the C/A SNP (*rs2071094*) typing data within the DMR-B (the *rs2071094* C allele has been converted into T allele after bisulfite treatment). The remaining five SNPs within the DMR-B were present in a homozygous status

fluorescently labeled forward primer and an unlabeled reverse primer flanking the highly polymorphic CAG repeat region and the two methylation sensitive *Hpa*II sites at exon 1 of *AR*, before and after *Hpa*II digestion (Fig. 2). The X-inactivation ratio was calculated using the area under curve after compensation for unequal amplification of the two alleles caused by the difference in the product size.

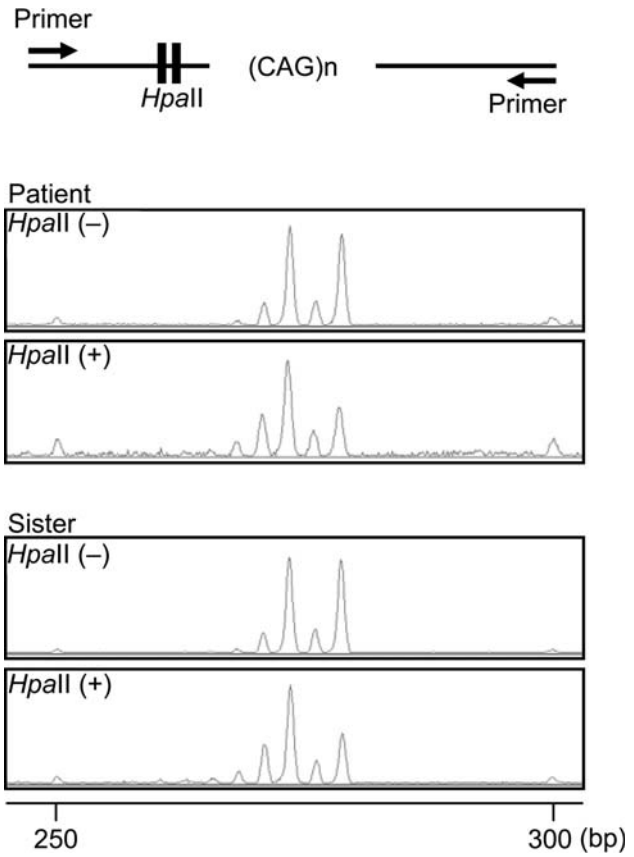

Fig. 2 X-inactivation analysis. The examined region contains a highly polymorphic CAG repeat region and two methylation sensitive *Hpa*II sites. Thus, PCR products are obtained from both active and inactive X chromosomes before *Hpa*II digestion and from inactive X chromosomes alone after *Hpa*II digestion. The comparison of the area under the *curves* between two heterozygous peaks (273 and 279 bp) before and after *Hpa*II digestion indicates that the two X chromosomes undergo random X-inactivation with nearly identical patterns between the twins. The small peaks are by-products caused by slippage phenomenon

Results

Microsatellite analysis

All the genotyping results were identical between the twins, indicating monozygosity (Table 1). Furthermore, upd(7)mat was excluded, as was upd(11p15)mat involving the *H19*-DMR.

Methylation analysis

The methylation indices of the patient (the DMR-A, 28.0% for the *Bsa*BI site and 30.4% for the *Mwo*I site; the DMR-B, 17.4% for the *Afl*III site and 17.1% for the *Hpy*188I site) were below the normal ranges (the DMR-A, 48.1–64.7% for the *Bsa*BI site and 50.1–67.6% for the *Mwo*I site; the DMR-B, 39.8–59.8% for the *Afl*III site and 39.3–58.0% for the *Hpy*188I site), whereas those of the twin sister, the father, and the mother were within the normal range (the DMR-A, 61.3, 61.7, and 62.3% for the *Bsa*BI site and 64.6, 64.9, and 61.0% for the *Mwo*I site; the DMR-B, 52.6, 52.7, and 51.5% for the *Afl*III site and 49.5, 50.4, and 52.9% for the *Hpy*188I site) (Fig. 1b). Furthermore, bisulfite sequencing revealed that the DMR-A and the DMR-B were predominantly hypomethylated in the patient and differentially methylated in the sister, although the SNP typing data for the seven SNPs were not informative for the parental origin of each clone in the twins (Fig. 1c). In the control subject, heterozygosity was detected for the T/G SNP (*rs2251375*) within the DMR-A and the C/A SNP (*rs2071094*) within the DMR-B, while the remaining five SNPs in the DMR-B were present in a homozygous status.

X-inactivation analysis

Both the patient and the twin sister had random X-inactivation with a nearly identical pattern (64%:36% for the patient and 65%:35% for the sister) (Fig. 2).

Discussion

We observed monozygotic twins discordant for SRS and identified hypomethylation of the *H19*-DMR (epimutation) in the affected twin only. In this regard, the clinical diagnosis of SRS in the affected twin was based on the criteria proposed by Hitchins et al. (2001), and the epimutation was clearly demonstrated for the two regions within the *H19*-DMR (DMR-A and DMR-B). Thus, although the difference in the clinical course might have some influence on the clinical and molecular discordance between the twins, the results imply that SRS in the affected twin is primarily caused by the epimutation occurring in the early fetal life.

The discordant methylation pattern of the *H19*-DMR may primarily be due to a failure to maintain the DNA methyltransferase-1 (DNMT1)-dependent methylation imprint around the pre-implantation S phase (Hirasawa et al. 2008). This notion has basically been suggested for monozygotic twins discordant for Beckwith–Wiedemann syndrome (BWS; MIM 130650) and hypomethylation of the KvDMR1 (Gaston et al. 2001; Weksberg et al. 2002). Indeed, such a failure would result in a hemimethylated daughter duplex that would subsequently be converted to a fully methylated and a fully unmethylated sister chromatid in the next S phase (Weksberg et al. 2002). This event would yield two different cell clones, one with normally methylated DMR with normal growth potential and the other with demethylated DMR with decreased growth potential, thereby leading to the separation of cells with different characters and resultant twinning discordant for

Table 1 The results of microsatellite analysis

Locus	Position	Mother	Patient	Sister	Father	Assessment
D3S1314	3q28	139	139/145	139/145	141/145	Biparental
D7S2846	7p14	177	177/181	177/181	181	Biparental
D7S519	7p13	261/263	261/263	261/263	261/263	NI
D7S1830	7p12	200	200/212	200/212	212	Biparental
D7S1870	7q11	118	118	118	118/120	NI
D7S634	7q21	136/144	138/144	138/144	126/138	Biparental
D7S527	7q21	291	291/295	291/295	273/295	Biparental
D7S1824	7q34	169/173	173	173	173	NI
D7S550	7q36	189/193	189	189	187/189	NI
D9S168	9p23	228/230	228/234	228/234	234/238	Biparental
D9S171	9p21	160/164	164/166	164/166	156/166	Biparental
D10S1580	10p12	140/148	140/150	140/150	142/150	Biparental
D10S198	10q24	183/197	183/197	183/197	183	NI
D10S1268	10q24	137/163	137	137	137	NI
D10S221	10q26	92/104	102/104	102/104	102	Biparental
D10S209	10q26	179	179/203	179/203	203	Biparental
D10S1700	10q26	125/127	125/127	125/127	123/127	NI
D11S2071	11p15	184/186	186/188	186/188	182/188	Biparental
D11S922	11p15	87/111	87	87	87	NI
D11S988	11p15	113	113/119	113/119	119/125	Biparental
D11S902	11p15	138/144	138/146	138/146	146/154	Biparental
D11S904	11p14	186/198	190/198	190/198	184/190	Biparental
D14S979	14q32	197/199	199/201	199/201	193/201	Biparental
D14S985	14q32	227/229	227/233	227/233	233/237	Biparental
D21S1446	21q22	206/222	206	206	206	NI
DXYS233	Xp22, Yp11	274/280	274/278	274/278	274/278	NI

NI not informative

The Arabic numbers indicate the PCR product sizes (bp). The primer sequences are available in the GDB Human Genome Database (http://www.gdb.org/)

SRS. Indeed, the methylation pattern of this patient would primarily be consistent with demethylation of the paternally derived *H19*-DMR in roughly half of cells. Although it might be possible that the *H19*-DMR hypomethylation took place after the twinning, this notion assumes no causal relationship between the *H19*-DMR hypomethylation and the twinning.

It may be worth pointing out that the twins were females. In this context, the sex ratio of previously reported five monozygotic twin pairs discordant for SRS is not remarkable with a male to female ratio of 2:3, and discordance for hypomethylation of the *H19*-DMR has been confirmed only in a single female twin pair (Nyhan and Sakati 1977; Samn et al. 1990; Bailey et al. 1995; Sagot et al. 1996; Gicquel et al. 2005). However, discordance for BWS and hypomethylation of the KvDMR1 is predominantly manifested by monozygotic female twins with a male to female ratio of 1:11 (Gaston et al. 2001; Weksberg et al. 2002). Since DNMT1 is also required for the maintenance of X-inactivation (Bestor 2000), this may have reduced the amount of DNMT1 enzyme available for the imprint maintenance of the DMRs, increasing the risk of demethylation of DMRs in the monozygotic female twins.

Hypomethylation of the *H19*-DMR was identified in leukocytes of this patient, but not in the twin sister. This contrasts the previous finding that *H19*-DMR in leukocytes is hypomethylated in both of the 11-year-old twin sisters discordant for SRS, whereas *H19*-DMR in skin fibroblasts is hypomethylated in the affected twin only (Gicquel et al. 2005). Such inconsistency may be due to the difference in placental vascular anatomy. While the present twins had no discernible interconnecting vascular anastomosis in the placenta, the twins described by Gicquel et al. (2005) may have shared fetal circulation, which allowed the transfer and engraftment of blood stem cells with demethylated *H19*-DMR from the affected twin to the unaffected twin. In this regard, since clinical features are similar between the

twins of this study and those reported by Gicquel et al. (2005), this implies that epimutations of the non-hematopoietic tissues such as skin fibroblasts are responsible for the development of SRS phenotype, whereas epimutations in leukocytes have no discernible clinical effects. For the present twins, since the X-inactivation pattern in leukocytes was nearly identical between the present twins in the probable absence of the shared fetal circulation, it may be that the X-inactivation pattern was established as a normal event simultaneously with, or just prior to, the demethylation of the *H19*-DMR as an abnormal event.

In summary, we identified monozygotic female twins discordant for SRS and the *H19*-DMR hypomethylation. This is reminiscent of hypomethylation of the KvDMR1 in one of the twin with BWS (Gaston et al. 2001; Weksberg et al. 2002). Since the *H19*-DMR and the KvDMR1 contiguously resides on chromosome 11p15, there may be a regional property for the occurrence of hypomethylation. Further studies will permit a better clarification of the prevalence of the monozygotic twins discordant for SRS and the mechanisms involved in this condition.

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