ORIGINAL ARTICLE

Quantitative analysis of methylation status at 11p15 and 7q21 for the genetic diagnosis of Beckwith–Wiedemann syndrome and Silver–Russell syndrome

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Methylation-specific (MS) multiplex ligation-dependent probe amplification (MLPA) at two differentially methylated regions (DMRs) at chromosome 11p15, *H19*-DMR and *LIT1*-DMR, and microsatellite analysis for uniparental disomy (UPD) at chromosome 7 or 11, have been recommended for the genetic diagnosis of the Beckwith–Wiedemann syndrome (BWS) and the Silver–Russell syndrome (SRS). In this study, the efficacy of the MS pyrosequencing method at *H19*-DMR and *LIT1*-DMR at 11p15 and *SGCE*-DMR at 7q21 was evaluated for the genetic diagnosis of BWS ($n=18$) and SRS ($n=20$) patients. Epigenetic alterations or UPD were detected in 83% of BWS and 50% of SRS individuals by MS-MLPA, but the detection rate increased to 95% of BWS and 70% of SRS by MS pyrosequencing. Thirteen BWS patients (72%) harbored loss-of-methylation (LOM) at *LIT1*-DMR and two patients (11%) harbored gain-of-methylation (GOM) at *H19*-DMR, whereas two patients (11%) had both LOM at *LIT1*-DMR and GOM at *H19*-DMR, reflecting paternal UPD 11. Thirteen SRS patients (65%) harbored LOM at *H19*-DMR, whereas one patient (5%) had GOM at *SGCE*-DMR, reflecting maternal UPD 7. Birth anthropometric profiles were significantly correlated to methylation scores at either *H19*-DMR or *LIT1*-DMR. In conclusion, MS pyrosequencing enhanced the detection rate of molecular defects in BWS and SRS. Moreover, it indicates that methylation status at 11p15.5 might have an important role in fetal growth.

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INTRODUCTION

Beckwith–Wiedemann syndrome (BWS; OMIM 130650) and Silver–Russell syndrome (SRS; OMIM 180860) are fetal growth disorders with opposite clinical characteristics. BWS is characterized by pre-and/or post-natal macrosomia, macroglossia, visceromegaly and exomphalos, and increased risk for embryonal tumors. By contrast, SRS is characterized by asymmetric intrauterine growth retardation without post-natal catch-up growth. Patients with BWS have characteristic facial features, including midfacial hypoplasia, ear crease and/or pit, and hemihyperplasia, whereas relative macrocephaly, triangular face, clinodactyly and facial or skeletal asymmetry are characteristic features of SRS. Most patients with BWS are developmentally normal, whereas ~30–40% of SRS patients exhibit developmental delay.^{1,2}

Epigenetic alterations at chromosome 11p15.5 are frequently demonstrated in both BWS and SRS patients. Two differentially

methylated regions (DMRs), *H19*-DMR and *LIT1*-DMR, exist in this region, having important roles in fetal and post-natal growth. The *H19*-DMR encompasses 2 kb upstream of the *H19* gene and is paternally methylated. It is expected to reciprocally regulate the expression of *H19* and *IGF2*. *H19* is maternally expressed and encodes an untranslated RNAPolIII transcript, which may act as a tumor suppressor.³ *IGF2* is a paternally expressed growth-promoting factor. On the other hand, the 5'-end of *KCNQ1OT1* is thought to function as the *LIT1*-DMR and is maternally methylated. It determines the expressions of two genes, *KCNQ1* and *CDKN1C*.⁴ *CDKN1C* is a strong inhibitor of cell proliferation.⁵

A paternal loss-of-methylation (LOM) at *H19*-DMR is found in over 50% of SRS patients,^{2,6} whereas a maternal gain-of-methylation (GOM) at *H19*-DMR occurs in 5–10% of BWS patients.^{1,7} At the *LIT1*-DMR, maternal LOM is identified in 50% of BWS patients, but

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no GOM has been reported in SRS patients.^{8,9} In addition, paternal uniparental disomy (UPD) encompassing 11p15.5 is found in 20%, and *CDKN1C* mutations in 5–10%, of BWS patients.^{9,10} In SRS, maternal UPD or duplication of 11p15.5 has been identified in a small number of patients.¹¹ Furthermore, maternal UPD of chromosome 7 is found in ~10% of SRS patients.² Maternal UPD 7 can be reflected by a GOM at the promoter region of the *SCGE* gene, which is located at 7q21 and maternally methylated.^{12,13} Recently, abnormal methylation, affecting multiple imprinting loci, has been identified in a subset of BWS and SRS patients.¹⁴

Up to 80% of BWS patients and 50–60% of SRS patients can be diagnosed genetically.^{1,2,11} However, rates of positive genetic testing can be influenced by the clinical diagnostic criteria used to enroll a patient, the testing methods applied and the ethnicity of the patients. Currently, methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) analysis is recommended as the first-line test for the genetic diagnosis of BWS and SRS, with microsatellite typing or molecular karyotyping also used in a subset of patients.^{1,2} However, because of the semiquantitative nature of the data that MS-MLPA yields, genetic diagnosis can be obscured by MS-MLPA when a patient has only partial or mosaic LOM or GOM. In this study, 38 Korean patients with BWS or SRS, exhibiting the typical clinical manifestations of the respective diseases, were tested for imprinting defects at chromosomal regions 11p15.5 and 7q21. Using the MS pyrosequencing method, we were able to positively identify epigenetic changes in more patients within our cohort compared with results obtained by the MS-MLPA analysis. This pilot study highlights that the MS pyrosequencing method improves the genetic diagnostic process for BWS and SRS, and is easily applicable as a routine clinical test.

MATERIALS AND METHODS

The Institutional Review Board of the Asan Medical Center, Seoul, Korea, approved the current study, and the parents of each patient provided informed written consent. Clinical diagnosis of BWS was based on the following clinical features: pre-natal and/or post-natal overgrowth (weight > the 90th percentile of age and gender-matched controls), hemihyperplasia, macroglossia, abdominal wall defect, ear anomalies, visceromegaly, renal abnormalities, cleft palate, positive family history, neonatal hypoglycemia, cardiomegaly and midfacial hypoplasia,^{15,16} whereas the diagnosis of SRS was based on intrauterine and post-natal growth retardation (weight < the 10th percentile of age and gender-matched controls), normal head circumference, fifth finger clinodactyly and triangular face.¹⁷ The patients presumed to have other alleged congenital disorders were excluded.

Methylation-specific multiplex ligation-dependent probe amplification

MS-MLPA was performed at *LIT1*-DMR and *H19*-DMR using the SALSA-MLPA kit, ME030 (MRC Holland, Amsterdam, The Netherlands) using 100 ng of genomic DNA, according to the manufacturer's instructions. Probes were hybridized to denatured genomic DNA and hybridized samples were divided into two separate tests; one ligated directly and the other digested with *HhaI*, an MS restriction enzyme, before ligation. After ligation, PCR was carried out with fluorescence-labeled unique primers for probe sets, including *LIT1*-DMR and *H19*-DMR regions. Amplified products were analyzed on an ABI3130xl Genetic analyzer (Applied Biosystems, Foster, CA, USA). Data analyses were performed using GeneMarker v.1.7 (SoftGenetics, Pittsburgh, PA, USA), with at least three normal control samples, according to the manufacturer's instructions.

MS pyrosequencing analysis

Bisulfite PCR and pyrosequencing primers were designed to amplify two CpG dinucleotide sites (nt. 7613 and nt. 7615) in the promoter of *H19* (AF087017.1), *H19*-DMR; five CpG dinucleotides (nt. 58813, nt. 58818,

nt. 58820, nt. 58823 and nt. 58827) in the 5'-end of *KCNQ1OT1* (NT_009237.18), *LIT1*-DMR; and five CpG dinucleotides (nt. 343, nt. 350, nt. 361, nt. 365 and nt.379) in the 5'-end of the *SCGE*-DMR (NT_007933.15) using PSQ Assay Design software v.1.0 (Qiagen, Hilden, Germany; Supplementary Table 1). Genomic DNA from peripheral leukocyte was treated with bisulfate solution, 2.5 M sodium bisulfate solved in 1 N NaOH with 20 mM hydroquinone (Sigma-Aldrich, St Louis, MO, USA) for 16 h at 50 °C water bath. To remove bisulfate and other salts, DNA was purified with column-type DNA purification kit, GeneAll (Generalbiosystems, Seoul, Korea).¹⁸ Bisulfite-modified DNA (20 ng) was amplified in a 25-μl reaction using a bisulfite-modified sequence-specific primer set and *Taq* polymerase (Solgent, Daejeon, Korea). Pyrosequencing was performed using the PyroGold kit and the PyroMark ID96 instrument (Qiagen), according to the manufacturer's instructions. Briefly, 20 μl of each biotinylated PCR product was immobilized on streptavidin-coated Sepharose HP beads (Amersham Biosciences, Piscataway, NJ, USA) and then subjected to sequencing using an automatically generated nucleotide dispensation order for 'sequence to analyze' corresponding to each reaction. Methylation indexes were calculated as the mean value of % mC (percent of methylated cytosine) for all examined CpGs interrogated, for *H19*-DMR, *LIT1*-DMR and *SCGE*-DMR in each sample. As an internal control, the methylation status of nonCpG cytosines was used to check for adequate bisulfite conversion. Reference range of methylation index at each DMR was defined as two s.d. below or above the mean value for 20 controls, who had normal growth profiles and who lacked clinical features of BWS and SRS.

Microsatellite analysis

PCR of the microsatellite markers, D11S4046 (11p15.5), D11S4146 (11p15.5), D11S1760 (11p15.4), D11S4190 (11p15.1), D11S899 (11p15.1), D11S902 (11p15), D11S909 (11p15), D11S904 (11p14.2), D11S914 (11p13), D11S935 (11p13), D11S1314 (11q13.4), D11S925 (11q23) and D11S968 (11q25) for chromosome 11; and D7S674 (7p12), D7S2429 (7p11), D7S663 (7p11), D7S630 (7q21), D7S657 (7q21), D7S501 (7q22), D7S662 (7q22), D7S523 (7q31), D7S486 (7p31), D7S2425 (7q33), D7S2465 (7q36) and D7S636 (7q36) for chromosome 7, was performed using a fluorescently labeled forward primer and conventional reverse primer. PCR products were run on an ABI3130xl Genetic analyzer (Applied Biosystems). Alleles were analyzed using GenMarker v.1.7 (SoftGenetics).

Statistical analyses

Statistical analyses were performed using SPSS for Windows (version 12.0; SPSS, Chicago, IL, USA). Continuous variables were analyzed using an unpaired Student's *t*-test and the Mann–Whitney *U*-test, whereas categorical variables were analyzed using Fisher's exact test. Correlations between methylation status (% mC) and growth profiles were analyzed using Spearman's rank correlation coefficient. Receiver operating characteristic curves were used to establish specific cut-off values for abnormal methylation at each locus analyzed by MS pyrosequencing in controls and patients. *P*-values less than 0.05 were considered to be significant.

RESULTS

Clinical characteristics of patients with BWS/SRS

A total of 18 patients with BWS and 20 patients with SRS were recruited. Macroglossia was noted in 94.4% (17/18 patients) of BWS patients. Other manifestations were variable among patients: macrosomia (61.1%); transient neonatal hypoglycemia (58.8%); ear anomalies, including ear crease or pit (50.0%); omphalocele (44.4%); renal anomalies, including hydronephrosis (33.3%) and multicystic dysplastic kidney (5.6%); intra-abdominal visceral organomegaly (27.8%); and hemihyperplasia (22.2%). Embryonal tumors were found in two BWS patients (11.1%), hepatoblastoma and Wilms tumor in individuals B8 and B16, respectively (Table 1 and Supplementary Table 2).

In SRS patients, dysmorphic features, including triangular-shaped face (100%), relative macrocephaly (100%), facial or limb asymmetry (90.0%) and fifth-finger clinodactyly (70.0%) were highly prevalent,

Table 1 Clinical characteristics of patients with BWS and their imprinting defects

Patient	Gender	Gestation (weeks)	Log ₁₀ (Bwt) (s.d. score)	Birth height (s.d. score)	Clinical findings	MS-MLPA	MS pyrosequencing	
							H19-DMR ^a	LIT1-DMR ^b
B1	Male	40	1.29	NA	M, S, R, N, DD	Normal	55.28	45.43
B2	Male	35	3.55	2.82	M, S, R, N	Normal	82.12	46.66
B3	Female	30	0.05	NA	S	LIT1-DMR (L)	51.10	1.86
B4	Female	32	3.44	4.86	M, S, E, H, R, N	LIT1-DMR (L)	55.42	1.96
B5	Female	27	1.78	3.23	M, S, E, H, R, N	LIT1-DMR (L)	54.29	4.02
B6	Male	28	1.25	NA	S, E	LIT1-DMR (L)	56.2	5.23
B7	Male	36	2.09	1.01	M, S, E	LIT1-DMR (L)	56.59	5.23
B8	Female	35	-0.69	0.25	M, S, E, H, P, R, T	LIT1-DMR (L)	50.00	5.47
B9	Female	38	1.29	4.13	M, S, H	LIT1-DMR (L)	54.57	7.68
B10	Male	32	1.97	0.84	H, R	LIT1-DMR (L)	52.36	7.85
B11	Male	36	4.02	-0.25	M, S, R	LIT1-DMR (L)	48.22	8.00
B12	Female	35	0.50	0.00	S, N	LIT1-DMR (L)	54.42	8.06
B13	Male	35	2.83	-0.35	S, E, H, N	LIT1-DMR (L)	48.91	9.44
B14	Female	35	3.70	-0.54	M, S, E, N	LIT1-DMR (L)	49.34	20.39
B15	Female	35	-0.63	0.00	S, H, N	normal	51.63	20.53
B16	Male	37	2.51	2.12	M, S, E, P, T, N	H19-DMR (G)	66.09	35.63
B17	Male	40	1.15	-0.32	S, P, N	LIT1-DMR (L)	78.13^c	16.33^c
B18	Female	31	2.32	-0.86	M, S, E, H, P	H19-DMR (G)/ LIT1-DMR (L)	90.17^c	9.23^c

Abbreviations: BWS, Beckwith–Wiedemann syndrome; DD, developmental delay; DMR, differentially methylated region; E, ear anomaly; (G), gain-of-methylation; H, umbilical or inguinal hernia; (L), loss-of-methylation; M, macrosomia; MS, methylation-specific; N, neonatal hypoglycemia; NA, not-available; P, hemihypertrophy; R, renal anomaly; S, macroglossia; T, embryonal tumor.

Bold character, abnormal methylation.

^aReference value (mean $\pm$ 2 s.d.), 50.1 $\pm$ 5.0 % mC (n = 20).

^b43.9 $\pm$ 6.02 % mC (n = 20).

^cPaternal uniparental disomy 11.

whereas only a small subset of patients had genitorenal anomalies, including hydronephrosis (10.0%), hypospadias (5.0%) and cryptorchidism (5.0%) (Table 2 and Supplementary Table 3). Developmental delay was noted in one BWS (6.7%) and seven SRS patients (21.4%).

The s.d. scores of birth, height and weight (presented as Log₁₀(birth weight in kg (Bwt, kg))) were calculated as previously described.¹⁹ The s.d. score of birth height was -3.1 ± 1.4 (-6.5 to -0.3) in SRS patients and 1.1 ± 1.8 (-0.9 to 4.9) in BWS patients ($P < 0.001$). The s.d. score of Log₁₀(Bwt) was -3.8 ± 1.5 (-6.2 to -0.3) in SRS patients and 1.8 ± 1.5 (-0.7 to 4.0) in BWS patients ($P < 0.001$).¹⁹

Genetic diagnosis of patients with BWS/SRS using the MS-MLPA method

MS-MLPA identified genetic defects in 15 of 18 BWS patients (83.3%) and 10 of 20 SRS patients (50.0%; Tables 1 and 2). In BWS patients, 13 (72.2%) had LOM at LIT1-DMR, 1 (5.6%) had GOM at H19-DMR and one (5.6%) had both LOM at LIT1-DMR and GOM at H19-DMR. All 10 SRS patients diagnosed by MS-MPLA (50%) had LOM at H19-DMR.

Genetic diagnosis of patients with BWS/SRS using the MS pyrosequencing method

The normal values of methylation (mean $\pm$ 2 s.d.) at H19-, LIT1- and SGCE-DMRs were 50.1 ± 5.0 % mC (n = 20), 43.9 ± 6.0 % mC (n = 20) and 60.1 ± 4.0 % mC (n = 20), respectively.

The rate of detection of genetic defects increased to 94.4% (17/18) in BWS and 70.0% (14/20) in SRS patients, when we applied the MS pyrosequencing method. In addition to the 12 BWS patients (66.7%) harboring LOM at LIT1-DMR and one patient (5.6%) with GOM at H19-DMR identified by the MS-MLPA method, this analysis revealed another patient (B2) with GOM at H19-DMR (Supplementary Figure 1). Moreover, two patients (B17–18; 11.1%) had both LOM

at LIT1-DMR and GOM at H19-DMR. In B17 (Table 1), only GOM at H19-DMR was identified by the MS-MLPA method (Supplementary Figure 1). Microsatellite marker studies confirmed that B17 and B18 harbored isodisomic paternal UPD at chromosome 11. In B18, the UPD spanned the whole of chromosome 11 (Figure 1a), whereas B17 exhibited segmental UPD of 11p13–15 (Figure 1b). For the remaining one BWS patient (B1, 5.6%), no genetic defects were detected in the H19- or LIT1-DMRs, and no CDKN1C mutation was identified.

Using the MS pyrosequencing test, three additional SRS patients were found to have LOM at H19-DMR, giving a total of 13 SRS patients (65.0%) exhibiting LOM at this locus (Supplementary Figure 1). To exclude the possibility that the results of the MS pyrosequencing method might not reflect UPD of chromosome 11 in some patients, microsatellite marker test at chromosome 11 was also performed in five BWS cases (B1–3, B5 and B16) and six SRS patients (S8, S10–11, S13, S15 and S17), who exhibited abnormal methylation patterns at either H19-DMR or LIT1-DMR alone; however, biparental heterodisomy was conserved in all of these 11 patients. MS pyrosequencing analysis at the SGCE-DMR was performed for seven SRS patients with normal results at the H19- and LIT1-DMRs. One patient (S20) had GOM at SGCE-DMR. Microsatellite marker studies confirmed that this patient harbored heterodisomic maternal UPD at chromosome 7 (Figure 1c). No methylation defect was observed at either chromosome 11p15.5 or 7 in the other six SRS patients (30.0%; Table 2).

The cut-off thresholds, determined from receiver operating characteristic curves, were 45.3 % mC for LOM at H19-DMR (100% sensitivity, 100% specificity, $P < 0.01$), 61.3 % mC for GOM at H19-DMR (100% sensitivity, 100% specificity, $P < 0.01$), 28.1 % mC for LOM at LIT1-DMR (100% sensitivity, 100% specificity, $P < 0.01$) and 80.9 % mC for GOM at SGCE-DMR (100% sensitivity, 100% specificity, $P = 0.09$).

Table 2 Clinical characteristics of patients with SRS and their imprinting defects

Patient	Gender	Gestation (weeks)	Log ₁₀ (Bwt) (s.d. score)	Birth height (s.d. score)	Clinical findings	MS-MLPA	MS pyrosequencing		
							H19-DMR ^a	LIT1-DMR ^b	SGCE-DMR ^c
S1	Female	38	-1.58	-3.3	M, F, A,C	Normal	48.44	42.5	59.70
S2	Female	40	-4.3	-2.43	M, F, A	Normal	50.39	43.81	54.74
S3	Female	26	-4.71	-5.61	M, F, A	Normal	51.48	47.48	58.15
S4	Male	37	-4.49	-6.52	M, F, A, DD	Normal	52.31	46.31	65.45
S5	Male	33	-1.49	-4.5	M, F, A,C, R, DD	Normal	56.38	43.3	57.99
S6	Female	37	-2.45	-1.8	M, F, A, DD	Normal	48.44	41.08	58.43
S7	Male	39	-5.11	-1.8	M, F, C	H19-DMR (L)	9.25	45.24	n.d.
S8	Female	39	-4.8	-3.3	M, F, A,C	H19-DMR (L)	13.07	46.75	63.13
S9	Male	37	-4.49	-1.79	M, F, R	H19-DMR (L)	14.27	44.82	60.84
S10	Female	36	-5.77	-0.29	M, F, A,C, DD	H19-DMR (L)	14.6	46.84	63.74
S11	Male	37	-3.64	-2.07	M, F, A,C, DD	H19-DMR (L)	17.7	44.00	57.48
S12	Female	40	-3.76	-3.34	M, F, A	H19-DMR (L)	22	45.51	59.92
S13	Female	40	-0.31	-3.59	M, F, A,C	H19-DMR (L)	22.71	44.42	n.d.
S14	Male	39	-3.84	-3.56	M, F, A,C, R	H19-DMR (L)	24.69	45.04	60.88
S15	Male	39	-5.33	-1.93	M, F, A,C, DD	H19-DMR (L)	26.49	44.76	61.53
S16	Male	40	-4.55	-3.4	M, F, A,C, R	H19-DMR (L)	27.23	43.63	60.17
S17	Male	36	-6.16	-4.19	M, F, A,C	Normal	29.83	44.37	60.41
S18	Male	38	-2.21	-2.3	M, F, A,C	Normal	34.16	44.74	61.32
S19	Male	39	-3.04	-3	M, F, A,C	Normal	42.34	43.27	61.54
S20	Male	33	-4.07	-3.53	M, F, A,C, DD	Normal	49.62	41.11	95.75^d

Abbreviations: A, facial or limb asymmetry; C, fifth finger clinodactyly; DD, developmental delay; DMR, differentially methylated region; F, triangular face; (L), loss-of-methylation; M, relative macrocephaly; MS, methylation-specific; n.d., not done; R, genitorenal anomaly; SRS, Silver-Russell syndrome.

Bold character, abnormal methylation.

^aReference value (mean $\pm$ 2 s.d.), 50.1 $\pm$ 5.0 % mC ($n=20$).

^b43.9 $\pm$ 6.02 % mC ($n=20$).

^c60.1 $\pm$ 4.0 % mC ($n=20$).

^dMaternal uniparental disomy 7.

Figure 1 Microsatellite marker analysis of chromosomes 11 and 7. The paternal uniparental isodisomy observed in patient B18 spanned the whole of chromosome 11 (a), whereas in B17 it was segmental (11p13–15) (b). In patient S20, maternal uniparental heterodisomy was identified spanning the whole of chromosome 7 (c). Gray areas indicate the region of UPD and their parental origin. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Correlation of growth profiles at birth to quantified methylation status at the H19- and LIT1-DMRs

Methylation scores at the H19- and LIT1-DMRs, quantified by MS pyrosequencing, were compared with the growth profiles of BWS or SRS patients at birth; birth weight (Log₁₀(Bwt) s.d. score) or birth height s.d. scores were compared with the methylation score (% mC) of H19- and LIT1-DMRs in each patient (Spearman's

rank correlation coefficient).¹⁹ A significant positive correlation was revealed between H19-DMR methylation score and Log₁₀(Bwt) (s.d.; $r_s=0.701$, $P<0.001$) or birth height (s.d.; $r_s=0.465$, $P=0.005$). A significant inverse correlation was also found between the LIT1-DMR methylation score and Log₁₀(Bwt) (s.d.; $r_s=-0.655$, $P<0.001$) and birth height (s.d.; $r_s=-0.647$, $P<0.001$; Figure 2).

Figure 2 Methylation score (% mC) of the *H19*-DMR was correlated to the s.d. score of Log₁₀(Bwt) ($r_s = 0.701$, $P < 0.001$) (a) and that of height ($r_s = 0.465$, $P = 0.005$) (b), whereas the methylation score (% mC) at *LIT1*-DMR was negatively correlated to the s.d. score of Log₁₀(Bwt) ($r_s = -0.655$, $P < 0.001$) (c) and height ($r_s = -0.647$, $P < 0.001$) (d). A full color version of this figure is available at the *Journal of Human Genetics* journal online.

The prevalence of clinical findings, including growth profiles, macroglossia, ear anomaly, hernia, hemihypertrophy, renal anomaly and embryonal tumor, was compared between BWS patients with and without LOM at the *LIT1*-DMR. No statistically significant association was observed; however, all three patients with LOM at the *LIT1*-DMR and GOM at the *H19*-DMR had hemihypertrophy. Furthermore, no significant differences in the frequencies of phenotypes, including relative macrocephaly, triangular face, facial or limb asymmetry, clinodactyly, genitorenal anomaly and developmental delay, were found between SRS patients with and without LOM at the *H19*-DMR (data not shown).

DISCUSSION

In this study we demonstrated that the MS pyrosequencing method increases the detection rate of genetic defects in BWS and SRS patients to 94 and 70%, respectively, from 83 and 50% using the MS-MLPA method. The major limitation of the MS-MLPA method is that the results are difficult to interpret when a patient shows a partial or mosaic methylation defects in the *H19*- or *LIT1*-DMRs. However, the MS pyrosequencing method can not only evaluate methylation pattern at each CpG site of *H19*- or *LIT1*-DMRs, but also it further identifies partial or mosaic methylation defects, enhancing their detection rates. Although all the CpG sites were not evaluated (two sites at *H19*-DMR and five sites at *LIT1*-DMR), the rates of positive identification of methylation defects using the MS pyrosequencing test reported here are higher than those reported in recent reviews.^{1,2}

The methylation scores of B1, B4, B6, B7, S5 and B16 were slightly out of the reference ranges, but they were not considered as abnormal. Instead, the cut-off thresholds for each DMR were statistically established, which have high sensitivity and specificity rates, and these values will be applicable to further analyses of other cases. Moreover, by evaluating both the *H19*- and *LIT1*-DMR regions, the results obtained by MS pyrosequencing analysis also reflect any UPD

at chromosome 11p15, as demonstrated in two of our patients with opposite methylation patterns at the *H19*- and *LIT1*-DMRs. Of 11 other BWS or SRS patients in this study carrying methylation defects in either *H19*- or *LIT1*-DMR alone, none were positive for UPD of chromosome 11p15. To confirm UPD at either chromosome 11p15 or at 7, microsatellite typing has been recommended.^{1,2} However, microsatellite marker analysis is time consuming and requires availability of DNA samples from both parents. In addition, the results are not informative when parents share the same copy number of a particular marker, and this can be a major obstacle to interpretation. However, our results using the MS pyrosequencing test indicate that a UPD test for chromosome 11p15 might be necessary only for patients showing opposite methylation defects at *H19*- and *LIT1*-DMRs.

Several epigenetic–phenotypic correlations in BWS or SRS have been described. The differential expression of *H19*, a tumor suppressor, is implicated in growth restriction.^{12,20,21} The overexpression of *IGF2* increases the risk of embryonal tumors in BWS.^{1,22} On the other hand, the pathogenic role of *KCNQ1*, a voltage-gated potassium channel, in the development of BWS is not known, whereas it would be logical if downregulated *CDKN1C*, by LOM at the maternal allele *LIT1*-DMR, is related to the development of macrosomia and tumors in BWS; however, only phenotypes such as hemihyperplasia, omphalocele and monozygotic twinning have previously been suggested to be related to LOM at *LIT1*-DMR.^{1,8,22} In our study these epigenetic–phenotypic correlations were not observed, which might be related to the small number of enrolled patients, most of whom with BWS demonstrated LOM at *LIT1*-DMR. Meanwhile, hemihyperplasia was highly prevalent in patients with both LOM at *LIT1*-DMR and GOM at *H19*-DMR, although the significance of this observation will require further evaluation in a larger patient cohort. Moreover, in SRS patients the absence of epigenetic–phenotypic correlations between patients with and without epigenetic changes or UPD of chromosome 7 is likely attributable to the very high prevalence of the major clinical findings.

Importantly, by using the MS pyrosequencing method our study revealed a direct association between alterations in methylation of *H19*-DMR and *LIT1*-DMRs, and fetal growth patterns. To correlate the quantified methylation status at *H19*- and *LIT1*-DMRs with growth profiles at birth, we calculated s.d. scores, which were matched both to gender and to gestational age at birth, using the data described by Niklasson *et al.*¹⁹ In that study, birth weight was log-transformed, Log₁₀(Bwt) s.d. score, to decrease the skewness for post-natal weight, in contrast to the height s.d. score. Interestingly, our test demonstrated highly significant correlations for both imprinting centers. Therefore, these results indicate that *H19*-DMR and *LIT1*-DMR might have critical roles in fetal growth.

For patients with BWS or SRS, showing normal methylation patterns at the *H19*- and *LIT1*-DMRs, we looked for mutations of *CDKN1C* in BWS and UPD of chromosome 7 in SRS patients. Although no *CDKN1C* mutations were found in our patients with BWS, this is consistent with reports from previous studies that they occur rarely (5–10% of BWS patients).^{9,10} Likewise, only one SRS patient was identified harboring UPD at chromosome 7, and this is also comparable to frequencies previously reported (3–15% of SRS patients).^{2,11} Recent reports by Bourque *et al.*²³ and Penaherrera *et al.*¹³ indicated a clear association between methylation status at *SGCE*-DMR and UPD of chromosome 7. In addition, from our simple quantification of methylation status at *SGCE*-DMR, the case with UPD 7 was easily identified. The methylation cut-off value for *SGCE* was not statistically significant, because the positive case was

Figure 3 Molecular diagnostic algorithm for patients with BWS or SRS. Parenthesis (%), detection rate in the current study. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

the only one among our SRS patients; however, the MS pyrosequencing test for *SGCE* could easily be applied to the diagnosis of maternal UPD of chromosome 7, as its results clearly demonstrate a fully methylated status for such cases.

The results of this study indicate that the molecular diagnostic approach for BWS and SRS patients can be further simplified compared with previous recommendations (Figure 3).^{1,2} The quantified methylation status at *H19*- and *LIT1*-DMRs at chromosome 11p15.5 can be initially investigated using the MS pyrosequencing method. The presence of UPD of chromosome 11 can be evaluated only for those patients with opposite methylation defects in the *H19*- and *LIT1*-DMRs. For patients with no methylation defects at the *H19*- and *LIT1*-DMRs, those with BWS should be investigated for mutations at *CDKN1C*, whereas for SRS patients an MS pyrosequencing test of the *SGCE*-DMR to assay for maternal UPD of chromosome 7 would be appropriate. Using this strategy, our mutation detection rate increased to 94.4% in BWS and 70.0% in SRS patients (Figure 3), although confirmation of the wider applicability of this strategy will require replication of our results in a larger cohort of BWS and/or SRS patients.

A small proportion of patients in this study with BWS or SRS remained genetically undiagnosed, despite this comprehensive diagnostic work-up. The phenotypic diversities of both BWS and SRS patients influence the process of clinical diagnosis of each disease, and this can also affect the rate of positive genetic testing. Currently, no consensual diagnostic criteria for BWS or SRS exist; however, all patients in our study for whom no genetic cause was identified manifested typical findings of their respective diseases, excluding the possibility of misdiagnosis. In addition, a low level of somatic mosaicism underlying these phenotypic differences can also affect the results of molecular testing, including the MS pyrosequencing test using DNA derived from peripheral blood. Moreover, genetic heterogeneity of BWS and SRS suggests that loci other than those at chromosome 11p15 can account for these diseases. A subset of patients with BWS and SRS have been reported to demonstrate methylation defects at other imprinted loci, including *ZAC1* (6q24),

GRB10 (7p12), *PEG1/MEST* (7q32), *IGF2R* (6q26), *GNAS* (20q13), *SNRPN* (15q11–13) and *DLK1/GTL2* (14q32), in addition to the *H19*- and *LIT1*-DMRs.^{14,24,25} Furthermore, the possibility that genes in nonimprinted regions may contribute to the development of BWS and SRS should be considered.²⁶ In this study, only methylation defects at the chromosomal regions 11p15 and 7q21 were evaluated. As a small subset of SRS patients (~1%) harbor segmental UPD or duplication at chromosome 7 not including 7q21,²⁷ these cases can be missed out by the quantification of methylation status at *SGCE*-DMR alone. Although not described in this report, we also investigated the methylation status at the *GRB10* (7p12) and *PEG1/MEST* (7q32) by MS pyrosequencing analysis;^{27,28} however, the methylation indexes of normal controls varied widely for these loci, precluding straightforward application of these tests to genetic testing in BWS or SRS (data not shown). For these cases with normal methylation indexes at 11p15 and 7q21, molecular karyotyping, such as chromosomal microarray or whole genome methylation assay, should be considered.^{2,13}

In conclusion, this pilot study indicates that quantitative investigation of imprinting defects at chromosomal regions 11p15 and 7q21 can simplify the molecular diagnostic process in the fetal growth disorders, BWS and SRS, as well as enhance the positive rate of identification of genetic defects. Considering the significant correlation of the methylation status of these regions with birth anthropometric profiles, the MS pyrosequencing method might be an effective tool in revealing epigenetic–phenotype correlations in these disorders, and this requires evaluation in a larger patient cohort.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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