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Deciphering Epigenetic Backgrounds in a Korean Cohort with Beckwith–Wiedemann Syndrome

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Background: Beckwith–Wiedemann syndrome (BWS) is a congenital overgrowth disorder caused by genetic or epigenetic alterations at two imprinting centers (ICs) in the 11p15.5 region. Delineation of the molecular defects is important for prognosis and predicting familial recurrence. We evaluated epigenetic alterations and potential epigenotype–phenotype correlations in Korean children with BWS.

Methods: Forty children with BWS with proven genetic or epigenetic defects in the 11p15.5 region were included. The phenotype was scored using the BWS consensus scoring system. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), bisulfite pyrosequencing, a single-nucleotide polymorphism microarray, and *CDKN1C* sequencing were used for confirmative diagnosis.

Results: Patients met the criteria for genetic testing, with a mean clinical score of 5.4±2.0. Methylation alterations were consistent between MS-MLPA and bisulfite pyrosequencing in all patients. Twenty-six patients (65.0%) had IC2 loss of methylation (IC2-LoM), 11 (27.5%) had paternal uniparental disomy (patUPD), and one (2.5%) had IC1 gain of methylation. Macroglossia and external ear anomalies were more common in IC2-LoM than in patUPD, and lateralized overgrowth was more common in patUPD than in IC2-LoM (all $P<0.05$). Methylation levels at IC2 were inversely correlated with birth weight standard deviation score ($r=-0.476$, $P=0.014$) and clinical score ($r=-0.520$, $P=0.006$) in the IC2-LoM group.

Conclusions: Comprehensive molecular analysis of the 11p15.5 region revealed epigenotype–phenotype correlations in our BWS cohort. Bisulfite pyrosequencing can help clarify epigenotypes. Methylation levels were correlated with fetal growth and clinical severity in patients with BWS.

Key Words: Beckwith–Wiedemann syndrome, Epigenetics, Imprinting centers, DNA methylation, Pyrosequencing, Korean

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INTRODUCTION

Beckwith–Wiedemann syndrome (BWS; OMIM#130650) is a rare congenital overgrowth disorder characterized by macrosomia, macroglossia, neonatal hypoglycemia, abdominal wall defects, lateralized overgrowth, and increased risk of embryonal tumors during early childhood [1]. The clinical manifestation of BWS is variable and spans a spectrum, including patients with a clinical diagnosis of BWS with or without a genetic or epigenetic alteration at the chromosome 11p15.5 imprinted region [2, 3]. Assisted reproductive technology (ART) has been suggested to disturb epigenetic reprogramming during gamete and em-

bryo development, leading to imprinting disorders (IDs), including BWS [4-6].

The chromosome 11p15.5 region harbors two differentially methylated regions (DMRs) that are regulated via its imprinting control regions: imprinting center (IC) 1 for the telomeric *H19/IGF2*:intergenic (IG)-DMR and IC2 for the centromeric *KCNQ1OT1*: transcription start site (TSS)-DMR [7]. Multiple genetic or epigenetic defects affecting the expression of imprinted genes within the 11p15.5 region are observed in nearly 80% of patients with BWS spectrum disorders: IC2 loss of methylation (IC2-LoM) in approximately 50%, paternal uniparental disomy (patUPD) in 20%, IC1 gain of methylation (IC1-GoM) in 5%–10%, *CDKN1C* mutation in 5%, and copy number variation (CNV) in <5% [2]. Methylation testing at both IC1 and IC2 is the primary molecular test to diagnose the BWS spectrum, and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), which concurrently detects methylation status and copy number, is most commonly used for initial diagnosis [2]. However, complex epigenetic etiology and the possibility of low-level mosaicism necessitate further molecular testing to identify epigenotypes.

Correlations between clinical phenotype and epigenotype, including different risks of embryonal tumors, have been reported [1, 8]. Embryonal tumors occur in approximately 8% of children with BWS spectrum, with different tumor risks according to the molecular subgroups (2.6%–28%), i.e., high risk in patients with an epigenetic defect involving the telomeric domain (IC1-GoM and patUPD) and low risk in patients with a defect affecting the centromeric domain (*CDKN1C* mutation and IC2-LoM) [8-10]. However, specific clinical outcomes vary in individual patients with BWS, likely due to somatic mosaicism and genetic or environmental background [11, 12]. Previous studies on BWS were mostly conducted in Western populations and were highly heterogeneous in terms of the clinical diagnosis of BWS [8, 11, 13, 14]. We conducted a comprehensive molecular analysis to identify the epigenetic backgrounds and potential epigenotype–phenotype correlations in Korean children with molecularly confirmed BWS.

MATERIALS AND METHODS

Subjects

In total, 59 patients visited Seoul National University Hospital, Seoul, Korea, between January 2010 and December 2021 that were clinically suspected of having BWS met the criteria for genetic testing (a clinical BWS score of ≥2). Forty (67.8%) patients were confirmed to have genetic or epigenetic defects and were enrolled in this retrospective study. All medical records, including birth history, serial growth status, and examination results, were collected. The phenotype was scored using the BWS consensus scoring system (Table 1) [2]. Age- and sex-specific SD scores (SDSs) for height, weight, and head circumference at birth and postnatally were assigned based on Fenton growth references and the 2017 Korean National Growth Charts, respectively [15,16]. Prenatal and postnatal overgrowth was defined as height or weight > two SDs of age- and sex-matched controls [2]. Lateralized overgrowth was defined as asymmetric overgrowth of body parts [17]. All participating parents provided written informed consent prior to study enrollment. This study was approved by the Institutional Review Board of Seoul National University Hospital (approval No.: 2106-120-1230).

Table 1. Consensus scoring system to define the BWS spectrum

Cardinal features (2 points per feature)	Suggestive features (1 point per feature)
1. Macroglossia	1. Birthweight >2 SDS above the mean
2. Exomphalos	2. Facial nevus simplex
3. Lateralized overgrowth	3. Polyhydramnios or placentomegaly
4. Multifocal or bilateral Wilms tumor or nephroblastomatosis	4. Ear creases or pits
5. Hyperinsulinism (lasting >1 week and requiring escalated treatment)	5. Transient hypoglycemia (lasting <1 week)
6. Pathology results: adrenal cortex cytomegaly, placental mesenchymal dysplasia, or pancreatic adenomatosis	6. Typical BWS spectrum tumors (neuroblastoma, rhabdomyosarcoma, unilateral Wilms tumor, hepatoblastoma, adrenocortical carcinoma, or pheochromocytoma)
	7. Nephromegaly or hepatomegaly
	8. Umbilical hernia or diastasis recti

For a clinical diagnosis of classical BWS, a patient requires a score of ≥4 (this clinical diagnosis does not require molecular confirmation of an 11p15.5 anomaly). Patients with a score of ≥2 (including those with classical BWS with a score of ≥4) merit genetic testing for BWS investigation and diagnosis.
Abbreviations: BWS, Beckwith–Wiedemann syndrome; SDS, SD score.

Genetic and epigenetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using a DNA isolation kit (Qiagen, Hilden, Germany). The DNA was used for MS-MLPA of the 11p15.5 region, followed by bisulfite pyrosequencing analysis in all individuals to quantify the average methylation level at IC1 and IC2 and validate the MS-MLPA results. If a CNV beyond the detection range of MLPA was suspected based on the MS-MLPA results, single-nucleotide polymorphism (SNP) microarray analysis was conducted. Sanger sequencing of *CDKN1C* was conducted for one patient who showed normal results in both MS-MLPA and bisulfite pyrosequencing (Fig. 1).

MS-MLPA was conducted using the SALSA MLPA kit (ME030 BWS/RSS; MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. Denatured genomic DNA was hybridized with probes and subjected to two separate tests: one involving direct ligation to identify CNV and one involving digestion with an *Hha*I restriction enzyme before ligation to detect the methylation status of the 11p15.5 region. After ligation, PCR was conducted using fluorescently labeled primers for the probes, including IC1 and IC2. The amplified products were separated using an ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed using the Coffalyser.Net software (MRC Holland), with at least three control samples for each test.

Bisulfite pyrosequencing was conducted using targeted assays covering four and seven consecutive CpG sites (CpGs) for IC1 and IC2, respectively (Supplemental Data Table S1). Genomic DNA was treated with sodium bisulfite using an EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA). After PCR amplification using the Hot-Start Taq Master Mix (Qiagen), pyrosequencing was conducted using a PyroMark Q24 pyrosequencer (Qiagen), following the manufacturer's instructions. Methylation levels were calculated as a percentage of methylated cytosine (mC) for each CpG site using PyroMark Q24 Software (v.1.0.10; Qiagen). The mean level of DNA methylation (%mC) at IC1 or IC2 in patients with BWS was compared with that in 20 age- and sex-matched controls with normal growth profiles. Patients with an average methylation level > or < two SDs from the mean of the controls were categorized as GoM or LoM, respectively.

SNP microarray analysis was conducted using the CytoScan Dx Assay (Thermo Fisher Scientific, Santa Clara, CA, USA), which contains 750,000 SNP probes encompassing most known OMIM and RefSeq genes. After data analysis using the Chromosome Analysis Suite Dx software (Thermo Fisher Scientific), the array data were assessed according to the guidelines for the interpretation and reporting of CNVs issued by the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen) [18].

Sanger sequencing of *CDKN1C* (NM_000076) was conducted

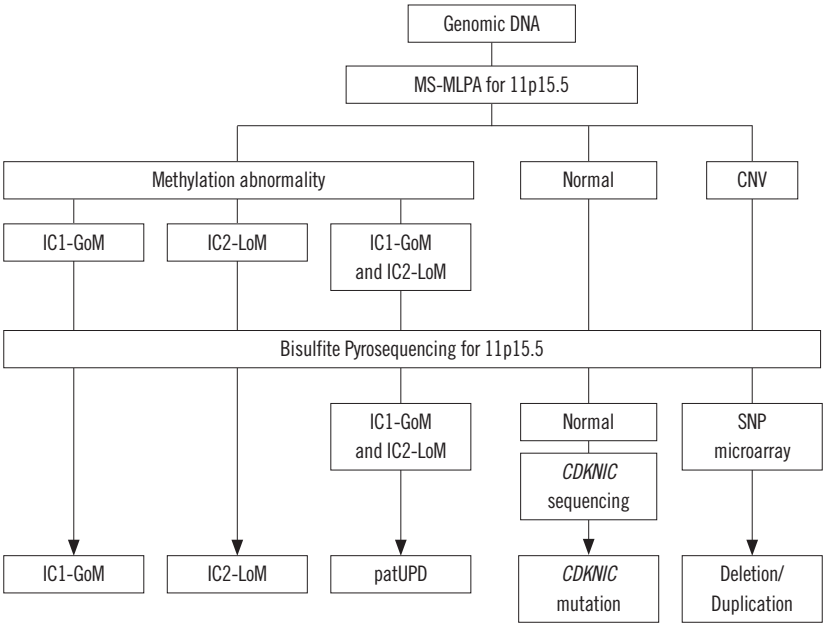

Fig. 1. Flowchart of molecular testing.
Abbreviations: MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; CNV, copy number variation; IC1-GoM, gain of methylation in imprinting center 1; IC2-LoM, loss of methylation in imprinting center 2; SNP, single-nucleotide polymorphism; patUPD, paternal uniparental disomy.

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for one patient who showed normal results in MS-MLPA and bisulfite pyrosequencing, to rule out the possibility of a *CDKN1C* point mutation. Two coding *CDKN1C* exons and exon–intron boundaries were PCR-amplified and sequenced using an ABI3730x1 Genetic Analyzer (Applied Biosystems) (Supplemental Data Table S2). Sequence variants were classified according to the ACMG guidelines [19].

Statistical analysis

Descriptive data are presented as the mean±SD and categorical variables as counts and proportions. Epigenotype–phenotype correlations for IC2-LoM and patUPD were investigated using the Mann–Whitney U test and Fisher's exact test. Statistical analysis was conducted using SPSS for Windows (v.25.0, IBM Corp., Armonk, NY). $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Table 2 shows the clinical and molecular characteristics of the 40 unrelated patients with BWS (20 boys and 20 girls). The mean age at first visit was 1.8±2.5 years, and the mean follow-up duration was 4.0±2.8 years. Six (15.0%) patients were conceived by ART; 15 (37.5%) were born preterm (<37 weeks of pregnancy), five cases of which were due to polyhydramnios or maternal preeclampsia. The mean SDSs of birth length, birth weight, and head circumference at birth were +1.1±1.4, +1.3±1.1, and +0.7±0.7, respectively. At the latest follow-up (at a mean age of 5.8±3.6 years), the mean SDSs of height, weight, and head circumference were +1.1±1.1, +1.0±1.3, and +0.0±0.0, respectively.

All patients met the criteria for genetic testing (a score of ≥2), with a mean clinical score of 5.4±2.0; 31 (77.5%) patients met the criteria for classical BWS (a score of ≥4). Macroglossia was the most frequent clinical feature observed in 27 (67.5%) patients, followed by lateralized overgrowth in 23 (57.5%) and ear creases or pits in 23 (56.1%). BWS-related pathologies were found in two patients (5.0%). Case 21 had nephrogenic rests in the left kidney, which was identified at the age of 1.2 months during tumor surveillance. Case 33 had diffuse nesidioblastosis related to hyperinsulinemia (distal pancreatectomy at 0.7 years of age) and bilateral adrenal hyperplasia causing premature adrenarche (left and right adrenalectomy at ages 6.9 and 8.0 years, respectively) (Table 2).

Epigenotype analysis

MS-MLPA identified epigenetic defects in 39 of the 40 patients (97.5%); 26 (65.0%) had IC2-LoM, one (2.5%) had IC1-GoM, 11 (27.5%) had both IC2- LoM and IC1- GoM, and one (2.5%) had CNV. One patient had a *CDKN1C* mutation. In case 27, IC1 and IC2 methylation defects were at marginal levels (Fig. 2A). Paternal duplication spanning the entire chromosome 11p15.5 as well as borderline methylation disturbances at both IC1 and IC2 (patUPD pattern) were revealed in the MS-MLPA results of case 39 (Fig. 2B). SNP microarray analysis confirmed that case 39 harbored a 4.0-Mb duplication at chromosome 11 (hg19, chromosome 11: 230,615–4,242,111) (Fig. 2C). Although molecular testing was not conducted for the parents, paternal duplication was suspected in case 39 because of the large size of the CNV, involving the entire domain, and opposite methylation changes in IC1 and IC2. A novel *CDKN1C* mutation was identified in case 40: c.688C>T (p.Q230*) (pathogenic; PVS1+PM2+PP5), which was inherited from his mother. Although paternal or maternal variant transmission could not be evaluated in the mother of case 40, she had a history of umbilical hernia repair operation, which suggested the possibility of familial BWS. Otherwise, there was no familial recurrence of BWS in our cohort.

Bisulfite pyrosequencing identified abnormal methylation in 39 of the 40 patients (97.5%), but not in the patient with a *CDKN1C* mutation (case 40). The reference methylation levels (mean±2 SD) in healthy controls (N=20) were 63.2±2.4 %mC at IC1 and 56.4±2.5 %mC at IC2. Case 27, who exhibited only marginal methylation defects at both IC1 and IC2 in MS-MLPA analysis, had both IC2-LoM (–4.4 SDS) and IC1-GoM (+1.7 SDS) and was confirmed as having patUPD [20]. Case 39, who exhibited CNV with borderline methylation defects of patUPD pattern in MS-MLPA analysis, showed concurrent methylation defects at both IC1 and IC2 by pyrosequencing (+2.5 SDS at IC1 and –7.3 SDS at IC2). Overall, 26 (65.0%) patients had IC2-LoM (–15.9±4.1 SDS), one (2.5%) had IC1-GoM (+6.3 SDS), and 12 (30.0%) had both IC2-LoM (–9.9±4.2 SDS) and IC1-GoM (+4.5±1.6 SDS). Methylation alterations were consistent between MS-MLPA and bisulfite pyrosequencing in all patients. Thus, the epigenotypes were classified as follows: 26 (65.0%) IC2-LoM, 11 (27.5%) patUPD, one (2.5%) IC1-GoM, one (2.5%) CNV, and one (2.5%) *CDKN1C* mutation (Table 2).

Epigenotype–phenotype correlations

Phenotypic features according to the two common molecular subtypes (IC2-LoM vs. patUPD) are presented in Table 3. All patients born after ART were IC2-LoM (26.1% vs. 0.0%, $P=0.011$).

Table 2. Clinical and molecular patient characteristics

No. case	Sex	Age (yr)	Gestation (weeks)	Length at birth (SDS)	Birth weight (SDS)	ART	Clinical score	Clinical manifestations		MS-MLPA (11p15.5)	Bisulfite pyrosequencing	
								BWS features	Other features		IC1 (%mC SDS)	IC2 (%mC SDS)
1	F	6.2	40	–0.3	+0.5	+	3	LO, E		IC2-LoM	N	L (–12.9)
2	M	1.5	28	+0.7	+2.1	-	7	S, O, MO, E	C, PDA	IC2-LoM	N	L (–20.4)
3	F	4.8	38	+1.7	+2.2	-	8	S, LO, MO, E, OM, U		IC2-LoM	N	L (–12.1)
4	M	6.1	33	+1.0	+1.7	-	8	S, O, P, E, T, OM	ASD, PS	IC2-LoM	N	L (–20.0)
5	F	6.2	37	+1.2	+0.9	-	6	S, LO, FN, E	PFO	IC2-LoM	N	L (–16.7)
6	M	3.9	37	NA	+2.0	-	5	S, FN, E, U		IC2-LoM	N	L (–19.6)
7	F	1.2	28	–1.6	–0.5	+	4	S, E, U	ASD	IC2-LoM	N	L (–10.2)
8	F	10.5	39	NA	+0.8	-	5	S, O, E	ASD	IC2-LoM	N	L (–19.3)
9	F	8.8	39	+0.6	+1.1	-	6	S, LO, FN, E	MMD	IC2-LoM	N	L (–11.8)
10	F	9.3	37	1.5	+2.5	-	8	S, O, LO, MO, T		IC2-LoM	N	L (–18.8)
11	M	7.1	39	NA	+1.3	-	8	S, O, LO, FN, P, OM		IC2-LoM	N	L (–19.0)
12	F	5.5	36	+0.6	+0.4	-	7	S, LO, FN, E, U		IC2-LoM	N	L (–15.0)
13	M	8.8	40	+0.9	+1.7	+	7	S, LO, FN, E, U		IC2-LoM	N	L (–16.9)
14	F	7.1	30	+3.2	+1.0	+	5	S, E, OM, U	C, PS	IC2-LoM	N	L (–20.5)
15	F	1.8	37	–0.1	+0.3	-	6	S, O, FN, E	PFO, PS	IC2-LoM	N	L (–18.9)
16	M	5.9	39	+4.0	+2.3	-	7	S, LO, MO, E, T		IC2-LoM	N	L (–15.2)
17	F	6.3	37	+1.6	+1.5	-	3	LO, FN		IC2-LoM	N	L (–7.7)
18	M	10.4	37	NA	+3.0	-	7	S, O, MO, P, E	PFO, VSD	IC2-LoM	N	L (–19.9)
19	F	1.1	33	+1.9	+1.7	-	5	S, O, E	ASD, PDA	IC2-LoM	N	L (–18.1)
20	M	1.1	37	NA	+2.2	+	4	MO, FN, E, U		IC2-LoM	N	L (–15.2)
21	F	3.2	37	NA	+1.2	+	5	S, R, E		IC2-LoM	N	L (–19.1)
22	M	15.8	30	NA	–0.6	NA	2	LO	HS, ADHD	IC2-LoM	N	L (–10.8)
23	M	14.7	40	NA	–1.3	NA	2	LO		IC2-LoM	N	L (–7.7)
24	F	8.9	33	NA	+0.6	NA	2	LO		IC2-LoM	N	L (–17.5)
25	M	0.6	31	NA	+2.2	-	6	S, O, MO, FN	Inguinal hernia	IC2-LoM	N	L (–18.3)
26	F	0.2	37	–0.2	+1.8	-	5	S, I, U		IC2-LoM	N	L (–10.9)
27	M	2.6	35	+0.6	+0.4	-	8	S, LO, I, FN, U		patUPD (marginal)	H (+1.7)	L (–4.4)
28	M	5.6	39	NA	–0.3	-	6	S, LO, E, U		patUPD	H (+5.0)	L (–8.7)
29	F	0.8	40	NA	+0.0	-	3	LO, U		patUPD	H (+2.0)	L (–3.0)
30	M	3.8	37	+1.8	+2.5	-	4	LO, MO, U		patUPD	H (+3.6)	L (–8.4)
31	M	8.9	39	+0.5	+0.9	-	5	S, LO, FN		patUPD	H (+5.6)	L (–15.3)
32	M	3.7	38	NA	+2.2	-	6	LO, MO, FN, E, OM	RC, FSGS	patUPD	H (+3.7)	L (–7.9)
33	F	8.5	34	NA	+1.2	-	7	I, A, NB, P, OM, U		patUPD	H (+7.0)	L (–17.3)
34	F	5.2	36	–0.8	+1.2	-	8	S, O, LO, E, T	ASD, PDA, GV	patUPD	H (+5.8)	L (–10.8)
35	F	5.0	40	–0.2	–0.9	NA	2	LO		patUPD	H (+5.2)	L (–11.1)
36	F	3.5	36	+0.9	+1.7	-	3	LO, U	VSD, PS	patUPD	H (+4.9)	L (–10.5)
37	M	6.8	39	+3.8	+1.6	-	2	LO		patUPD	H (+5.2)	L (–11.7)
38	M	8.0	37	+2.9	+2.8	-	8	S, MO, P, E, T, OM, U	VSD, AP	IC1-GoM	H (+6.3)	N
39	M	5.4	35	NA	+4.4	-	5	S, MO, P, OM		CNV (dup)*	H (+2.5)	L (–7.3)
40	M	5.2	36	+0.6	+1.1	-	7	S, O, FN, E, OM	PS, RC, BU	N†	N	N

*A 4.0-Mb duplication was detected at chromosome 11p15.5p15.4; †*CDKN1C* mutation was revealed in this case.

Abbreviations: SDS, SD score; ART, assisted reproductive technique; BWS, Beckwith–Wiedemann syndrome; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; IC1, imprinting center 1; IC2, imprinting center 2; F, female; LO, lateralized overgrowth; E, ear anomalies; LoM, loss of methylation; N, normal; L, low; M, male; S, macroglossia; O, exomphalos; MO, macrosomia; C, cleft palate; PDA, patent ductus arteriosus; OM, organomegaly; U, umbilical hernia and/or diastasis recti; P, polyhydramnios and/or placentomegaly; T, transient hypoglycemia; ASD, atrial septal defect; PS; pulmonary stenosis; FN, facial nevus simplex; PFO, patent ductus arteriosus; NA, not available; MMD, moyamoya disease; VSD, ventricular septal defect; R, nephrogenic rest; HS, hypospadia; ADHD, attention deficit hyperactivity disorder; I, hyperinsulinism; patUPD, paternal uniparental disomy; H, high; RC, renal cyst; FSGS, focal segmental glomerulosclerosis; A, adrenal hyperplasia; NB, nesidioblastosis; GV, genu valgum; AP, arched palate; GoM, gain of methylation; CNV, copy number variant; dup, duplication; BU, bifid uvula.

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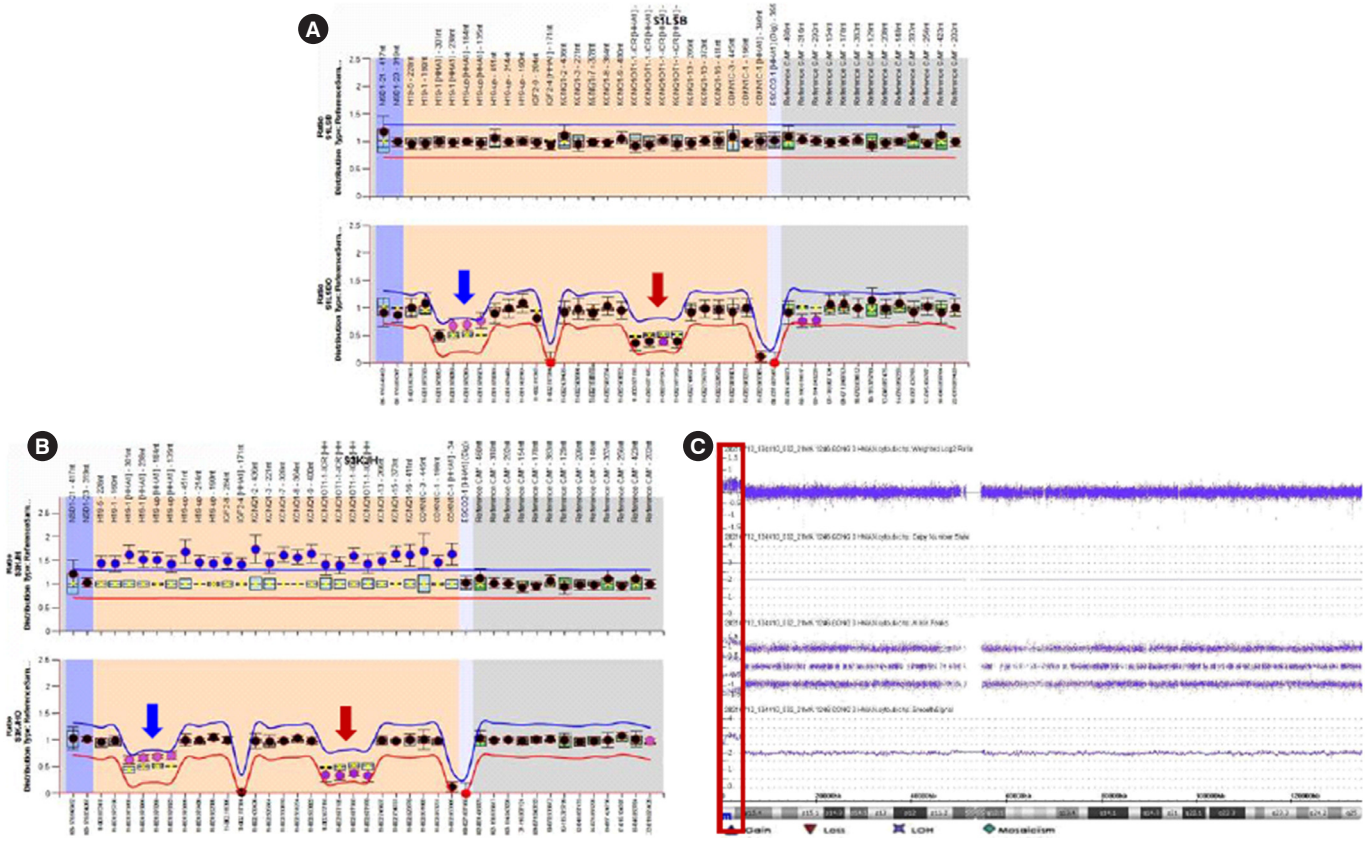

Fig. 2. Results of epigenotype analysis in two patients. For MS-MLPA analysis, copy number and methylation status in each individual were presented in upper and lower panel, respectively. (A) Ratio chart for MS-MLPA analysis for 11p15.5 region in case 27, showing concomitant presence of IC1 hypermethylation (blue arrow) and IC2 hypomethylation (red arrow), both at marginal levels (in lower panel). (B) Ratio chart for MS-MLPA analysis for 11p15.5 region in case 39, demonstrating duplication spanning the entire chromosome 11p15.5 (in upper panel) with borderline methylation disturbances at both IC1 and IC2 (blue and red arrows in lower panel), which suggested 11p15.5 paternal duplication. (C) SNP microarray analysis revealed a 4.0-Mb duplication (hg19, chromosome 11: 230,615–4,242,111) in case 39 (red box).
Abbreviations: MS-MLPA, methylation-specific-multiplex ligation-dependent probe amplification; IC1, imprinting center 1; IC2, imprinting center 2; SNP, single-nucleotide polymorphism.

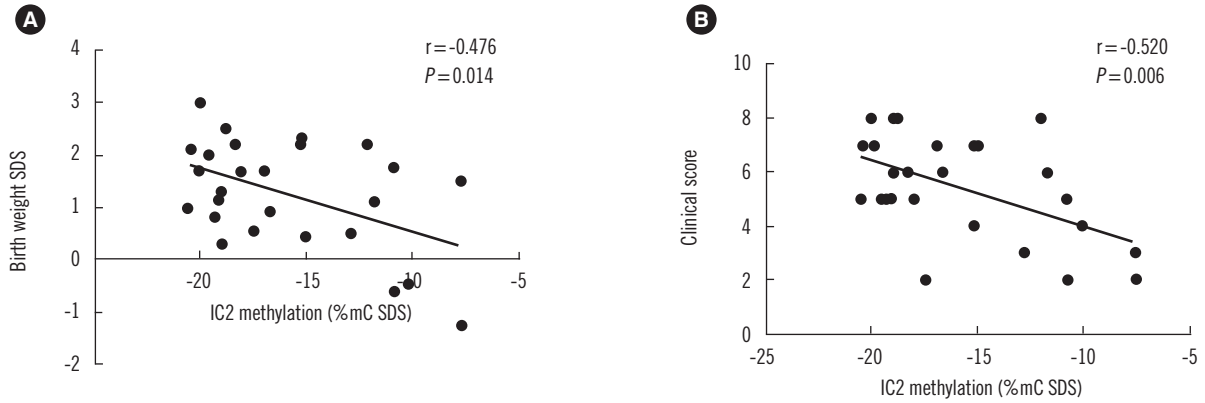

Fig. 3. Correlations between methylation level at IC2 and clinical parameters in the IC2-LoM group. (A) Inverse correlation between IC2 methylation and birth weight SDS (r=–0.476, P=0.014). (B) Inverse correlation between IC2 methylation and clinical score (r=–0.520, P=0.006).
Abbreviations: IC2, imprinting center 2; LoM-IC2, loss of methylation at IC2; %mC, percentage of methylated cytosine; SDS, SD score.

Table 3. Clinical characteristics by molecular subtype

Characteristics	Overall (N=40)	IC2-LoM (N=26)	patUPD (N=11)	*P*
ART, N (%)	6 (15.0)	6 (26 1)	0 (0.0)	0.011
Paternal age at conception (yr)	37.8±4.5	39.3±4.7	35.1±3.3	0.029
Maternal age at conception (yr)	34.5±4.2	35.5±4.6	32.9±2.9	0.129
Birth length (SDS)	+1.1±1.4	+1.1±1.3	+0.9±1.5	0.871
Birth weight (SDS)	+1.3±1.1	+1.2±1.0	+1.0±1.1	0.431
Head circumference at birth (SDS)	+0.7±0.7	+0.6±0.6	+0.7±0.7	0.840
Age at last visit (yr)	5.8±3.6	6.0±4.2	4.9±2.4	0.424
Height at last visit (SDS)	+1.1±1.1	+1.2±1.1	+0.4±0.8	0.030
Weight at last visit (SDS)	+1.0±1.3	+1.0±1.4	+0.6±1.0	0.301
Head circumference at last visit (SDS)	+0.0±0.0	+0.0±0.0	+0.0±0.0	0.670
Clinical score	5.4±2.0	5.4±1.9	4.9±2.3	0.485
Macroglossia, N (%)	27 (67.5)	20 (76.9)	4 (36.4)	0.028
Exomphalos, N (%)	11 (27.5)	9 (34.6)	1 (9.1)	0.224
Lateralized overgrowth, N (%)	23 (57.5)	13 (50.0)	10 (90.9)	0.019
Wilms tumor or nephroblastomatosis, N (%)	1 (2.5)	1 (3.8)	0 (0.0)	1.000
Hyperinsulinism, N (%)	3 (7.5)	1 (3.8)	2 (18.2)	0.205
Pathology*, N (%)	1 (2.5)	0 (0.0)	1 (9.1)	0.297
Macrosomia (birthweight > 2 SDS), N (%)	11 (27.5)	7 (26.9)	2 (18.2)	0.695
Facial nevus simplex, N (%)	14 (35.0)	11 (42.3)	3 (27.3)	0.477
Polyhydramnios or placentomegaly, N (%)	7 (17.5)	6 (23.1)	1 (9.1)	0.649
Ear creases or pits, N (%)	23 (57.5)	18 (69.2)	3 (27.3)	0.030
Transient hypoglycemia, N (%)	5 (12.5)	3 (11.5)	1 (9.1)	1.000
Nephromegaly or hepatomegaly, N (%)	8 (20.0)	3 (11.5)	2 (18.2)	0.623
Umbilical hernia or diastasis recti, N (%)	15 (37.5)	8 (30.8)	6 (54.5)	0.173

Data are presented as the mean±SD or N (%).
*This included adrenal cortex cytomegaly, placental mesenchymal dysplasia, or pancreatic adenomatosis.
Abbreviations: ART, assisted reproductive technology; IC2-LoM, loss of methylation of imprinting center 2; patUPD, paternal uniparental disomy; SDS, SD score.

The height SDS at the latest follow-up was significantly higher in the IC2-LoM group than in the patUPD group (1.2±1.1 vs. 0.4±0.8, P=0.030). Paternal age was higher in the IC2-LoM group than in the patUPD group (39.3±4.7 vs. 35.1±3.3, P=0.029), but this difference became insignificant once patients born after ART were excluded. The IC2-LoM group showed a higher prevalence of macroglossia (76.9% vs. 36.4%, P=0.028) and external ear anomalies (69.2% vs. 27.3%, P=0.030) than the patUPD group. Conversely, the patUPD group showed a higher prevalence of lateralized overgrowth (50.0% vs. 90.9%, P=0.019) than the IC2-LoM group. No significant intergroup differences were observed in the prenatal growth parameters and clinical scores. Regarding BWS-related pathologies, nephrogenic rests were found in one IC2-LoM patient (case 21), whereas one patient with patUPD (case 33) developed two different pathologies in the pancreas and adrenal glands requiring surgery.

Methylation levels at IC1 and IC2, as quantified by bisulfite pyrosequencing, were compared with prenatal and postnatal growth profiles or clinical scores. In the IC2-LoM group, methylation levels at IC2 showed significant inverse correlations with birth weight SDS (r=–0.476, P=0.014) and clinical score (r=–0.520, P=0.006) (Fig. 3). Interestingly, case 33, who developed two different BWS-related pathologies, showed the highest IC1 methylation level (+7.0 SDS) (Table 2).

DISCUSSION

We conducted a conclusive molecular diagnosis of the 11p15.5 region in a Korean BWS cohort using complementary techniques. Although MS-MLPA is currently the most common diagnostic

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test [2], other quantitative techniques, such as bisulfite pyrosequencing, can help in diagnosing patients with low-level mosaicism, especially in case of patUPD [21, 22], as revealed in our study. Moreover, chromosomal rearrangements, a rare condition with a familial recurrence risk of 50%, may also cause IC1 or IC2 dysregulation. Chromosomal rearrangements and *CDKN1C* mutations need to be investigated not only in familial cases but also in sporadic cases with normal methylation and copy number. In a subcategory of the BWS spectrum, such as isolated lateralized overgrowth, the diagnostic rate can be improved by analyzing other tissues to confirm tissue mosaicism, as revealed in our previous study [23].

We identified IC2-LoM and patUPD in 65.0% and 27.5% of 40 Korean patients with BWS, respectively. Epigenotype–phenotype correlations were identified for the two common epigenotypes, and they were grossly similar to those previously reported in other BWS cohorts [3, 10, 24-27]. In particular, macroglossia and external ear anomalies were associated with IC2-LoM and lateralized overgrowth with patUPD. In our BWS cohort with confirmed epimutation, BWS-related pathologies were observed in two (5.0%) patients (one IC2-LoM and one patUPD), without any typical BWS spectrum tumors, such as Wilms tumor or hepatoblastoma. In BWS, the tumor risk and tumor types differ according to the molecular subgroups [8-10, 24]. The tumor risk was high in IC1-GoM (28%) and patUPD (16%), with frequent Wilms tumors, whereas patients with IC2-LoM showed low tumor risk (2.6%) and usually developed other tumors, such as hepatoblastoma, rhabdomyosarcoma, and neuroblastoma [8]. The IC2-LoM frequency and relatively short follow-up duration in our BWS cohort may have influenced the results on tumor occurrence in the present study.

Like in Western countries, live births of ART-conceived infants have dramatically increased in Korea; in 2011, 2.83% of all births were associated with financial support from the National Supporting Program for the Subfertile [28]. ART has been related with IDs, although it is unclear whether ART itself or the genetic background of infertile parents is associated with epigenetic disturbances [4, 5]. In Italy, the relative risk of developing BWS was approximately 10-fold in children conceived by ART when compared with the general population [6]. The frequency of ART-conceived patients (15.0%) in our BWS cohort with confirmed epimutation, studied between 2010 and 2021, was higher than the 4.0% frequency in a French BWS cohort with epigenetic defects reported in 2003 [29]. The higher frequency of patients conceived by ART in our study possibly reflects a selection bias and may also reflect an increase in awareness of BWS over the years over which our study was conducted.

Our patients who conceived through ART were exclusively IC2-LoM, which is consistent with results in previous reports. Previously, IC2-LoM was described in 11 out of 12 patients with BWS born after ART [29, 30]. Another study identified IC2-LoM in 24 out of 25 patients with BWS conceived through ART [31]. These observations provide evidence of a causal link between ART and IC2-LoM, implying that either ART itself or the genetic background of infertility may damage methylation acquisition or maintenance at the maternally imprinted region at 11p15.5 [32]. As parents get older, environmental exposures may influence post-transcriptional histone modifications and methylation patterns [33]. In addition, delayed maternal childbearing has been suggested to be associated with the development of maternal UPD 15 owing to increased non-disjunction at maternal meiosis 1 [34]. In our study, the difference in paternal age between the IC2-LoM and patUPD groups seemed to be biased as paternal age was higher in the subgroup of IC2-LoM who conceived through ART. The effects of ART procedures on the epigenetic profile require further investigation.

Considering the possibility of mosaic epigenetic alterations leading to mild methylation defects, we investigated the relationships between methylation levels and BWS features to clarify epigenotype–phenotype correlations. In patients with IC2-LoM, IC2 methylation levels in peripheral blood lymphocytes were correlated with birth weight and clinical severity. In a study on Silver–Russell syndrome and BWS, birth weight and length were positively correlated with IC1 methylation levels but inversely with IC2 methylation levels [21]. Another study on BWS also reported significant correlations between the methylation percentage at IC1 and the BWS phenotype: severe GoM (75%–86%) was associated with macroglossia, macrosomia, and visceromegaly, and mild GoM (55%–59%) with abdominal wall defects [22]. Notably, case 33, who showed the highest IC1 methylation level, developed two different BWS-related pathologies, suggesting a possible relationship between IC1 hypermethylation and the risk of pathology.

This study had some limitations. First, selection bias may exist because the patients were enrolled in a single tertiary center. In addition, clinical information, such as birth length and birth head circumference, were missing for some participants, which may have influenced the statistical significance of the results for these parameters. Second, we could not confirm patUPD of entire chromosome 11 using microsatellite analysis or SNP-based chromosome microarray analysis, although mosaic segmental UPD(11)pat is probable when both IC1-GoM and IC2-LoM are

detected without evidence of CNV [20]. Genome-wide patUPD may affect up to 10% of patients with UPD and needs to be considered in UPD patients, especially in patients with additional clinical features and unusual cancer predisposition [35]. Third, owing to the retrospective study design, we could not evaluate the presence of chromosomal aberrations (e.g., balanced translocation) in the asymptomatic parents of the patient with CNV. Fourth, the relatively short follow-up duration restricted the evaluation of long-term growth outcomes, including final adult height. Finally, a subset of patients with BWS show aberrant methylation patterns, described as multi-locus imprinting disturbance (MLID), affecting imprinted loci other than the disease-specific 11p15.5 region [36]. While most reported patients with MLID exhibited clinical features of the original ID only, MLID testing may help to interpret the phenotypic divergence in the BWS spectrum. The strength of this study lies in that we conducted a comprehensive phenotypic evaluation and molecular analysis of the disease-specific locus in a Korean BWS cohort with proven genetic or epigenetic defects.

In conclusion, we found epigenotype–phenotype correlations in our BWS cohort. Quantitative bisulfite pyrosequencing at IC1 and IC2 can help clarify the epigenotype in 11p15.5, and methylation levels seem to correlate with phenotypic severity. Further studies are warranted to fully understand the pathophysiological consequences of BWS and their epigenetic disturbances.

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AUTHOR CONTRIBUTIONS

Kim HY and Ko JM designed the study and were involved in clinical evaluation. Shin CH, Lee YA, Shin CH, and Kim G-H contributed to the data acquisition. Kim HY drafted the manuscript. Shin CH and Ko JM supervised the study. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

None declared.

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Supplemental Data Table S1. Primers used for bisulfite pyrosequencing

Gene	Gene location	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Sequencing primer (5′ to 3′)
IC1 (*H19/IGF2*)	11p15.5	AGGGTTTAAGGGGGTTATTTG	TAACTTAAATCCCAAACCATAACA	TTATGGGAGTAGTATTAGATTTTT
IC2 (*KCNQ10T1*)	11p15.5	GTGATGTGTTTATTATT	TGGAGGTTTGTGGGYGTTTAG	GTGATGTGTTTATTATT

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Supplemental Data Table S2. Primers used for Sanger sequencing of *CDKN1C*

CDKN1C	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)
Exon1_1	CGTTCCACAGGCCAAGTGCG	GCTGGTGCGCACTAGTACTG	374
Exon1_2	CGTCCCTCCGCAGCACATCC	CCTGCACCGTCTCGCGGTAG	279
Exon1_3	TGGACCGAAGTGGACAGCGA	AGTGCAGCTGGTCAGCGAGA	496
Exon1_4	CCGGAGCAGCTGCCTAGTGTC	CTTTAATGCCACGGGAGGAGG	539
Exon2	CGGCGACGTAAACAAAGCTG	GGTTGCTGCTACATGAACGG	469