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Epigenetic mosaicism and cell burden in Beckwith–Wiedemann syndrome due to loss of methylation at imprinting control region 2

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Abstract Beckwith–Wiedemann syndrome (BWS) is a rare overgrowth disorder caused by epigenetic alterations on Chromosome 11p15.5. Most molecular changes are sporadic and are thought to occur in a mosaic pattern. Thereby, the distribution of affected cells differs between tissues for each individual, which can complicate genotype–phenotype correlations. In two of the BWS molecular subtypes, tissue mosaicism has been demonstrated; however, mosaicism has not been specifically studied in the most common cause of BWS, loss of methylation (LOM) at *KCNQ1OT1:*TSS differentially methylated region (DMR) imprinting center 2 (IC2) LOM. The increased prevalence of twinning associated with the IC2 LOM subtype and the discordant phenotypes between the twins previously led to the proposal of diffused epigenetic mosaicism, leading to asymmetric distribution of affected cells during embryonic development. In this study, we evaluated the level of methylation detected in 64 samples collected from 30 individuals with IC2 LOM. We demonstrate that the IC2 LOM defect can occur in mosaic and nonmosaic patterns, and tissues from the same individual can show variable patterns, which suggests that this asymmetric distribution occurs during development. We further suggest that the clinical phenotype in individuals with BWS IC2 LOM is correlated with the epigenetic burden of affected cells in each tissue type. This series is the first report to demonstrate tissue mosaicism within the IC2 LOM epigenotype, and consideration of this mosaicism is necessary to understanding the pathogenesis of BWS.

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INTRODUCTION

Beckwith–Wiedemann syndrome (BWS; OMIM 130650) is the most common overgrowth disorder affecting ~1 in 10,340 live births (Mussa et al. 2013). The use of assisted reproductive technologies (ARTs) increases this frequency to 1 in 1100 (Mussa et al. 2017). The phenotype of BWS is composed of a variety of congenital malformations as well as presenting sequelae such as macroglossia, omphalocele, lateralized overgrowth, hypoglycemia, and increased risk for development of certain tumors, most often kidney (Wilms' tumor) or liver (hepatoblastoma). An international consensus group defined BWS as a spectrum, rather than as a

syndrome, to describe the range of clinical manifestations and molecular abnormalities associated with the BWS region on Chromosome 11p15 (Brioude et al. 2018).

The Beckwith–Wiedemann spectrum (BWSp) can be further conceptualized as two separate spectrums. The molecular spectrum describes the range of epigenetic alterations affecting Chromosome 11p15.5 that can cause BWSp: loss of methylation at *KCNQ1OT1*:TSS-DMR (IC2 loss of methylation [LOM]), gain of methylation (GOM) at *H19/IGF2*:IG-DMR1 (IC1 GOM), and paternal uniparental isodisomy of Chromosome 11 (pUPD11). Rarer molecular subtypes include *CDKN1C* variants or Chromosome 11p15 anomalies (deletions, duplications, translations, etc.), which can be inherited. The phenotypic spectrum describes the various clinical presentations and issues that can affect individuals and are likely caused by the molecular alterations present. These concepts can be bridged through epigenotype–phenotype associations, which provide insight into the specific roles that the IC1 and IC2 regions play in the development of BWSp manifestations.

Molecular diagnosis in individuals affected by BWSp can be challenging because of the presence of mosaicism, in which the burden of affected cells can vary in a single individual. Within BWSp, mosaicism is most often associated with the pUPD11 subtype (Kalish et al. 2016; Brioude et al. 2018; Baker et al. 2021a). Furthermore, mosaic IC1 GOM has also been demonstrated, with the molecular defect only detected in tissue samples such as wild type (WT) or a skin biopsy (MacFarland et al. 2018; Baker et al. 2021a). Individuals affected by pUPD11 or IC1 GOM often present with more variable phenotypes compared to those affected by IC2 LOM, because of this mosaicism (Duffy et al. 2019). The IC2 LOM phenotype is most often associated with classic BWSp features such as macroglossia, omphalocele, ear creases/pits, and facial nevus simplex; in addition, this subgroup has a lower risk for tumors compared to other molecular subgroups (Brioude et al. 2018; Duffy et al. 2019).

An increased prevalence of female twins affected by IC2 LOM has been associated with BWSp, and twins often present with discordant phenotypes (Weksberg et al. 2002; Bliek et al. 2009; Cohen et al. 2019). Diffused epigenetic mosaicism has been proposed to describe the phenotypic divergence that can occur between twins affected by BWSp (Cohen et al. 2019). More recently, case reports have provided support for this theory (Fontana et al. 2020; Sun et al. 2021); however, no direct evidence demonstrating the tissue mosaicism exists to date.

In this study, we examined samples collected from individuals affected by IC2 LOM and compared the methylation levels detected in tissues with the levels detected in the blood. We additionally evaluated the phenotypes of the individuals to determine whether the degree of IC2 LOM correlated with specific features.

RESULTS

Clinical Presentation

A total of 64 samples collected from 30 individuals underwent 11p15.5 methylation analysis in a single laboratory as described (Baker et al. 2021a,b). There were 13 females (43.3%) and 17 males (56.7%). Eight individuals (27.5%) were conceived via ART procedures; conception history was unavailable for one individual. The majority of individuals were from singleton pregnancies ($n = 26$), and four individuals (13.3%) were part of multiple gestation pregnancies (two monozygotic [MZ] and two dizygotic [DZ]). The two DZ pregnancies were conceived via ART, and the affected individuals were males; both of their female twin sisters did not have features of BWSp. One MZ individual was a male triplet from a pregnancy classified as monochorionic–diamniotic (MC-DA), and he was the single triplet in the amniotic sac; both twin siblings were unaffected. The other MZ individual was a MC-DA female twin at birth, and the pregnancy was originally a triplet gestation with fetal demise in the first

trimester. Her twin sister (not included in this study) showed concordant IC2 LOM levels in the blood, with discordant BWSp phenotype.

Genomic Analyses

A positive IC2 LOM result was detected in at least one sample from all 30 individuals, and positive results were detected in 61 of 64 samples (95.3% overall positivity rate); all samples demonstrated normal IC1 methylation levels. We considered the methylation results from each sample as the "IC2 LOM profile" and defined the blood sample profile as the "constitutional LOM profile" for each individual, and two groups were classified: constitutional (blood positive) and tissue-mosaic (blood negative). The methylation percentage detected in each sample was categorized into three groups: (1) COMPLETE LOM (c-LOM) was defined as >95% of cells affected by IC2 LOM (or <2.5% methylation detected in IC2 region); (2) PARTIAL LOM (p-LOM) was defined as between 5% and 95% of cells affected by IC2 LOM (or between 2.5% and ~47.5% methylation detected in the IC2 region); or (3) NORMAL (<2.5% of cells affected by IC2 LOM, normal variation).

A constitutional IC2 LOM profile was established for the majority of individuals, with more than half affected by c-LOM in blood (Table 1). Four individuals were classified as "tissue-mosaic IC2 LOM": three individuals had negative blood analysis with positive IC2 LOM detected in tissue (tissue-mosaic); one additional individual was classified as tissue-mosaic, with an unknown constitutional profile (Table 1). The results for each individual and samples in the cohort are available in Supplemental Table S1.

Mosaic and nonmosaic IC2 LOM results were demonstrated in the group (Fig. 1). Similar distributions of c-LOM and p-LOM constitutional profiles were established (blood); however, tissue profiles were more mosaic in some individuals (Fig. 1). Within the constitutional IC2 LOM subgroup ($n = 26$), three mosaic distribution groups were observed: seven individuals had c-LOM in all tissues evaluated and were considered constitutional c-LOM IC2; eight individuals were observed to have discrepant degrees of IC2 LOM (mosaic LOM IC2), with c-LOM most often detected in blood and p-LOM detected in the other sample; and 11 individuals had p-LOM in all samples evaluated and were considered constitutional p-LOM IC2 (Fig. 1). Extremely low levels of p-LOM were detected in the tissue samples from the four individuals with tissue-mosaic IC2 LOM (Fig. 1); tissues included two tongue samples and two skin biopsy samples from an affected region.

The highest rate of c-LOM was found in blood samples, and this sample type also had the lowest average methylation percentage (Table 1). Matched blood sample comparison was available for 29 individuals. The sample with the highest number of affected cells (i.e., lowest IC2 LOM methylation level detected) was most often blood ($n = 22$); however, close to one-quarter of individuals were found to have a tissue with lower methylation levels than blood ($n = 4$ tongue; $n = 3$ skin; $n = 1$ prenatal [singleton]).

In individuals with a constitutional IC2 LOM profile established ($n = 26$), the degree of mosaicism (or variation) was considered the difference in methylation levels measured in blood and the other samples tested. The average variation within each individual was 8.5% ± 7.8%, which corresponds to one sample affected by ~15% more or less IC2 LOM cells compared to the other sample. More than half of these individuals ($n = 14$) were affected by >5% variation, corresponding to a >10% difference in the number of affected cells; and close to one-third ($n = 8$) were affected by >10% variation, corresponding to a >20% difference in affected cell populations.

Six individuals had prenatal testing performed, with postnatal blood confirmation detected in all cases; and the prenatal and postnatal IC2 LOM levels were within 5% variation in more than half of the individuals (Supplemental Table S2). The three individuals conceived via ART were observed to have higher variations detected (13% average variation)

Table 1. Characteristics of the patients and samples in the cohort

Total Patients ($n = 30$)	Frequency, % (n)
LOM classification	
Constitutional IC2 LOM	86.7 (26)
Tissue mosaic IC2 LOM	13.3 (4)
Constitutional LOM degree ($n = 26$)	
Complete IC2 LOM (c-LOM)	26.9 (7)
Mosaic IC2 LOM	30.8 (8)
Partial IC2 LOM (p-LOM)	42.3 (11)
Total samples ($n = 64$)	
Positive testing	95.3 (61)
Average % IC2 ($n = 61$)	10.53 ± 12.47
Blood sample ($n = 29$)	
Positive testing	89.7 (26)
Average % IC2 ($n = 26$)	4.39 ± 5.61
c-LOM	57.7 (15)
Tongue sample ($n = 22$)	
Positive testing	100 (22)
Average % IC2	14.36 ± 13.46
c-LOM	22.7 (5)
Skin sample ($n = 7$)	
Positive testing	100 (7)
Average % IC2 ($n = 7$)	20.56 ± 19.80
c-LOM	28.6 (2)
Prenatal testing[a] ($n = 6$)	
Positive testing	100 (6)
Average % IC2 ($n = 6$)	11.42 ± 8.71
Matched samples available	$N = 29$ patients
Blood—tongue	22 patients
Blood—skin biopsy	6 patients
Blood—prenatal[a]	6 patients

(c-LOM) Complete loss of methylation, (IC2) imprinting control region 2 (*KCNQ1OT1:* TSS DMR), (LOM) loss of methylation, (p-LOM) partial loss of methylation.
[a]Amniocentesis (cultured cells $n = 3$; direct $n = 3$).

compared to the three naturally conceived pregnancies (2.7% average variation). The MZ pregnancy showed a lower variation between samples compared to the DZ pregnancy. Prenatal amniocyte type (cultured vs. direct) did not appear to differ, with the average variation between 5% and 10% for both groups. Two individuals were affected by variations of >10% comparing blood and prenatal samples—one individual with cultured amniocyte testing and one individual with direct amniocyte testing; both of these individuals were conceived via ART. In four individuals with prenatal testing, tongue samples were also analyzed. The two individuals who were conceived via ART were observed to have more variation between the prenatal testing, blood, and tongue testing, whereas similar levels between the three sample types were detected in the two naturally conceived individuals (Supplemental Table S2).

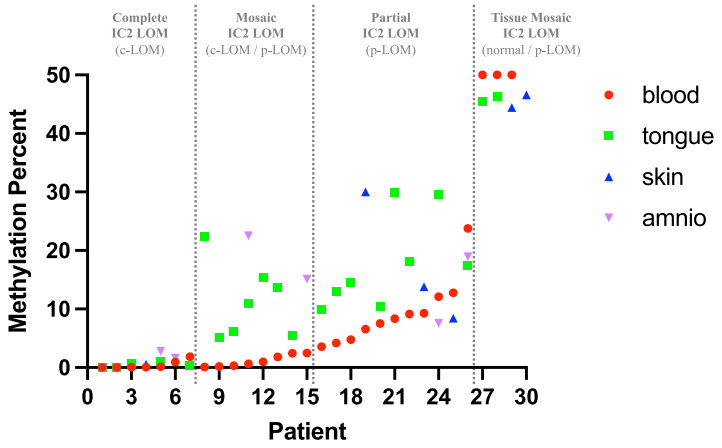

Figure 1. Methylation levels at IC2 measured in blood and other samples collected from 30 affected individuals. The methylation percent measured at IC2 for each sample type and each individual are visually represented in the figure. Within the constitutional IC2 loss of methylation (LOM) subgroup, four mosaic distribution groups were observed (dashed lines are used to demarcate the groups): seven individuals had COMPLETE LOM (c-LOM) in all tissues evaluated and were considered constitutional c-LOM IC2 (Individuals #1–7); eight individuals were observed to have discrepant degrees of IC2 LOM (mosaic LOM IC2), with c-LOM most often detected in blood and PARTIAL LOM (p-LOM) detected in the other sample (Individuals #8–15); and 11 individuals had p-LOM in all samples evaluated and were considered constitutional p-LOM IC2 (Individuals #16–26). Extremely low levels of p-LOM were detected in the tissue samples from the individuals with tissue-mosaic IC2 LOM (Individuals #27–30); tissues included two tongue samples and two skin biopsy samples from an affected region. Blood samples are represented by red circles; tongue samples are represented by green squares; skin biopsy samples are represented by blue triangles (facing up); and prenatal testing through amniocentesis samples (amnio) are represented by purple triangles (facing down). c-LOM was classified as methylation levels <2.5% (corresponding to >95% of IC2 LOM cells in the sample); p-LOM was classified as methylation levels between 2.5% and 47.5% (corresponding to 5%–95% of IC2 LOM cells in the sample); and negative testing (no LOM) was classified as methylation levels between 47.5% and 52.5% (normal population variation). All methylation testing was performed in single laboratory, with specific methodology available in the study by Baker et al. 2021a.

Phenotypic Analyses

Constitutional IC2 LOM Phenotype

Clinical characteristics and BWSp phenotypic features of the individuals with constitutional IC2 LOM ($n = 26$) are presented in Table 2. Individuals affected by c-LOM were observed to have higher BWSp clinical scores (i.e., a more pronounced phenotype), whereas individuals affected by p-LOM tended to have lower clinical scores (i.e., a less pronounced phenotype; Table 2). Individuals affected by partial LOM or mosaic LOM (those with c-LOM and p-LOM profiles detected in samples) were observed to have more variably affected phenotypes (i.e., a wider range in clinical scores; Table 2). In individuals affected by constitutional IC2 LOM, the BWSp clinical score appeared to be correlated with the level of IC2 methylation (Fig. 2). Pearson correlation testing demonstrated a significant relationship of lower IC2 methylation levels to higher BWSp clinical scores ($r = -0.543$, $P = 0.004$).

Omphalocele was most common in the c-LOM group and least common in the p-LOM group. Minor abdominal wall defects (AWDs) were seen in individuals with mosaic or p-LOM; and two individuals with p-LOM were not affected by any AWDs (Table 2). All individuals were affected by macroglossia with the exception of two individuals with p-LOM. The rate of hypoglycemia was similar among the groups; however, the severity of hypoglycemia differed: hyperinsulinism (HI), the more severe form, was frequent in the c-LOM group; transient hypoglycemia was frequent in the mosaic LOM group, and similar rates of HI and transient

Table 2. Clinical characteristics between mosaic IC2 LOM distribution groups in patients with constitutional *KCNQ1OT1:*TSS DMR loss of methylation ($n = 26$)

			Mosaic degree of IC2 LOM		
Clinical characteristics	Total constitutional IC2 LOM, % (*n*)	Constitutional complete IC2 LOM (c-LOM), % (*n*)	Partial IC2 LOM (p-LOM) ,% (*n*)	Mosaic IC2 LOM (c-LOM/p-LOM), % (*n*)	Complete IC2 LOM (c-LOM), % (*n*)
Number of patients	26	57.8 (15)	42.3 (11)	30.8 (8)	26.9 (7)
Patient sex					
Female	42.3 (11)	46.7 (7)	36.4 (4)	50 (4)	42.9 (3)
Male	57.7 (15)	53.3 (8)	63.6 (7)	50 (4)	57.1 (4)
Conception, ART	32.0 (8/25)	33.3 (5)	30 (3/10)	37.5 (3)	28.6 (2)
Twin/multiple gestation	11.5 (3)	6.7 (1)	18.2% (2)	12.5 (1)	(0)
Preterm (<37 wk)	50.0 (13)	53.3 (8)	45.5 (5)	37.5 (3)	71.4 (5)
Macroglossia	92.3 (24)	100 (15)	81.8 (9)	100 (8)	100 (7)
Tongue reduction	87.5 (21/24)	66.7 (10)	88.9 (8/9)	100 (8)	71.4 (5)
Abdominal wall defects	92.0 (23/25)	100 (14/14)	81.8 (9)	100 (8)	100 (6/6)
Omphalocele	57.7 (15)	66.7 (10/15)	45.5 (5)	50 (4)	85.7 (6/7)
Minor AWD	32.0 (8/25)	28.6 (4/14)	36.4 (4)	50.0 (4)	0/6
Lateralized overgrowth	68.0 (17/25)	73.3 (11)	60 (6/10)	62.5 (5)	85.7 (6)
Hypoglycemia	80 (20/25)	86.7 (13)	70 (7/10)	87.5 (7)	85.7 (6)
Severe (hyperinsulinism)	42.3 (11)	46.7 (7)	36.4 (4)	25.0 (2)	71.4 (5)
Transient hypoglycemia	36.0 (9/25)	40.0 (6)	30 (3/10)	62.5 (5)	14.3 (1)
BWSp clinical score	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Total BWSp score	8.69 ± 2.15	9.73 ± 1.62	7.27 ± 2.00	9.00 ± 1.51	10.58 ± 1.40
Range (min–max)	8 (4–12)	5 (7–12)	7 (4–11)	4 (7–11)	4 (8–12)
Cardinal features (number)	2.58 ± 0.95	2.87 ± 0.99	2.18 ± 0.75	2.38 ± 0.92	3.43 ± 0.79
Range (min–max)	3 (1–4)	3 (1–4)	2 (1–3)	3 (1–4)	2 (2–4)
Suggestive features (number)	3.54 ± 1.42	4.00 ± 1.13	2.90 ± 1.58	4.25 ± 1.28	3.71 ± 0.95
Range (min–max)	7 (0–7)	5 (2–7)	6 (0–6)	4 (3–7)	3 (2–5)

(LOM) Loss of methylation, (DMR) differentially methylated region, (ART) assisted reproduction technology, (AWD) abdominal wall defect, (BWSp) Beckwith–Wiedemann spectrum, (max) maximum, (min) minimum, (SD) standard deviation.

hypoglycemia were seen in the p-LOM group (Table 2). No apparent differences in sex distribution were observed, and ART conception was present in approximately one-third of the cohort. Twin gestation was observed only in the mosaic and p-LOM subgroups (Table 2).

Tissue-Mosaic IC2 LOM Phenotype

Four individuals were classified as tissue-mosaic IC2 LOM, with a positive result identified in an affected tissue and a negative result in blood. These individuals presented with variable phenotypes, and their tissue profiles showed <15% of cells affected by IC2 LOM (Table 3).

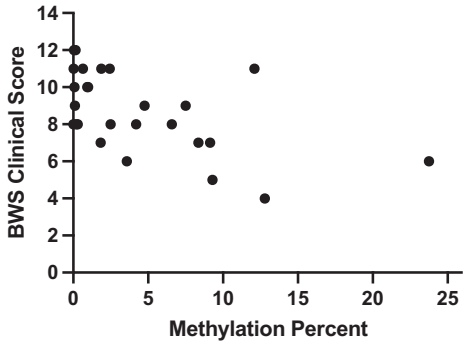

Figure 2. Scatterplot of Beckwith–Wiedemann spectrum (BWSp) clinical score by IC2 methylation level detected in blood samples from 26 affected individuals with IC2 loss of methylation (LOM). Individuals with lower methylation levels tended to have higher BWSp clinical scores. Pearson correlation testing demonstrated a significant inverse correlation ($r = -0.543$) between total BWSp score and IC2 methylation level ($P = 0.004$).

DISCUSSION

In this study, we demonstrate that the distribution of IC2 LOM can occur in mosaic and non-mosaic patterns, and the degree of affected cells appears to correlate with phenotype. Whereas mosaicism in blood is common within BWSp and IC2 LOM, this study represents the first description of IC2 LOM mosaicism in non–blood tissues derived from the affected individuals. To understand the etiology of this cell distribution and related phenotypes, we need to consider the timing of IC2 LOM and the tissue-specific burden of the IC2 LOM cells.

Table 3. Phenotype of tissue-mosaic IC2 LOM patients (low-level mosaicism detected in a tissue sample)

	Patient 27 (male)	Patient 28 (female)	Patient 29 (female)	Patient 30 (male)
Methylation				
Blood profile	No LOM	No LOM	No LOM	-
IC2 methylation	~50%	~50%	~50%	-
LOM-affected cells	<5% (normal)	<5% (normal)	<5% (normal)	-
Tissue profile	p-LOM	p-LOM	p-LOM	p-LOM
IC2 methylation	45.50%	46.41%	44.42%	46.61%
LOM-affected cells	~9% (low-level)	~7% (low-level)	~11% (low-level)	~7% (low-level)
Source[a]	Tongue	Tongue	Skin biopsy	Skin biopsy
Phenotype				
Clinical score	5 points	9 points	3 points	3 points
Cardinal features	M + LO (mild)	M + LO	LO	LO
Suggestive features	Nephromegaly	LGA, FNS, Org, Hyp, P	Ear crease (slight)	Umbilical hernia
BWSp group	"Atypical"	"Classic"	"Isolated LO"	"Isolated LO"
Additional features				
Prenatal	Triplet (MC-DA)	-	Pre-eclampsia	-
Other features	-	-	Facial dysmorphism (mild) LLD	Inguinal hernia LLD (mild)

(LOM) Loss of methylation, (FNS) facial nevus simplex, (Hyp) transient hypoglycemia, (ILO) isolated lateralized overgrowth, (LGA) large for gestational age, (LLD) leg length discrepancy, (LO) lateralized overgrowth, (M) macroglossia, (MC-DA) monochorionic–diamniotic, (NP) not performed, (Org) organomegaly, (P) pregnancy findings (polyhydramnios and/or placentomegaly), (TR) tongue reduction, (BWSp) Beckwith–Wiedemann spectrum.
[a]Tongue samples collected during TR procedure (#27–28); skin biopsy samples collected from larger LO leg (#29), and surgical site of umbilical hernia repair (#30).

Timing of IC2 LOM

Understanding of epigenetic regulation of the 11p15 region affected in BWSp is largely based on studies in mouse models of the largely conserved region. Establishment of the epigenetic marks occurs in the gametes, and these marks are maintained through the genomic reprogramming necessary during fertilization, the transition from haploid to diploid, and normal embryonic development (Weaver et al. 2009; Kalish et al. 2014; Chang and Bartolomei 2020). A combination of factors appears to mediate maintenance in mice (Kalish et al. 2014; Chang and Bartolomei 2020). Temporal disruption occurring between the establishment and maintenance of epigenetic marks can lead to loss of methylation (Ginart et al. 2016). Although mouse models can be used to study the epigenetic regulation of the 11p15 region, they do not provide the complete human complement of the BWS clinical phenotypic spectrum.

Monozygotic twins provide insight into potential embryologic timing of the IC2 LOM defect. Some have suggested that the methylation failure leading to IC2 LOM precedes the twinning process and that the IC2 LOM may in fact trigger the twinning process itself (Bliek et al. 2009; Cohen et al. 2019; Fontana et al. 2020). Fontana et al. suggested that the establishment of X-inactivation (XCI) and the twinning process precede the event(s) that lead to IC2 LOM and multilocus imprinting disturbance (MLID), and that a "wave of demethylation" may occur after XCI establishment as a result of observing nonhomogeneous IC2 and MLID patterns in samples collected from a pair of MZ twins (Fontana et al. 2020). In contrast, Sun et al. suggested that IC2 LOM occurs before twinning and may be a result of DNMTO1 failure in the context of ART conception, at least in MZ, dichorionic–diamniotic twins (Sun et al. 2021). More rarely, mutations in maternal-effect genes have been identified (Brioude et al. 2018). The potential influence of hematopoietic stem cell (HSC) contribution within individuals has additionally been hypothesized (Bliek et al. 2009; Sun et al. 2021). Regardless of the specific timing of the event, the theory of diffused epigenetic mosaicism (Cohen et al. 2019) has been consistently supported. However, given that the epigenetic deregulation spans beyond IC2 because of large global processes, further research to understand the extra-IC2 roles is needed.

Tissue Distribution in IC2 LOM: Diffused Epigenetic Mosaicism

In both twins and singletons affected by IC2 LOM, mosaic and nonmosaic patterns can occur, and variable patterns are observed among tissues collected from the same individual. We considered the results from the blood sample analyses as the constitutional epigenotype profile for each individual. We found that the IC2 LOM was detectable in blood for most individuals; however, some individuals were affected by IC2 LOM detectable in affected tissues, but not blood. These observations support the theory of diffused mosaicism and provide insight into the timing of when the IC2 LOM may have occurred during embryonic development (Table 4).

In individuals with constitutional IC2 LOM profiles, for some individuals, it appeared that all tissues are affected by the same degree of LOM in cells (suggesting nonmosaic distribution), whereas other individuals had a mosaic distribution of affected cells among tissues, leading to distinct levels of IC2 LOM detected between samples (Table 4). In individuals affected by later constitutional IC2 LOM defects, it is possible that not all tissues will have IC2 LOM, leading to the lack of associated phenotypic features; this remains theoretical as all samples analyzed from individuals in this subgroup were positive in the present study.

Individuals can additionally have IC2 LOM detected in an affected tissue in the context of a negative constitutional profile (tissue mosaic distribution). In these individuals, it is likely that the event occurred during the cell migration/distribution process, leading to a more

Table 4. Theories of diffused mosaicism and epigenetic burden

	Nonmosaic IC2 LOM	Mosaic IC2 LOM			Tissue mosaic IC2 LOM
	Very early	Early ←		→ late	
Embryologic timing	Event happened early in embryogenesis resulting in all cells (or nearly all cells) in the blood affected by IC2 LOM	Event happened early in embryogenesis resulting in all cells (or nearly all cells) in the blood affected by IC2 LOM	Diffused mosaicism occurred during embryogenesis, resulting in some cells in the blood affected by IC2 LOM	Diffused mosaicism occurred during embryogenesis, resulting in some cells in the blood affected by IC2 LOM	Diffused mosaicism occurred later during embryogenesis, resulting in no cells in the blood affected by IC2 LOM
Burden in blood	Complete (c-LOM)	Complete (c-LOM)	Partial (p-LOM)	Partial (p-LOM)	Not affected
Migration and distribution to tissues	Likely occurred before migration/ distribution, leading to all cells (or nearly all cells) in tissue affected by IC2 LOM	Likely occurred before migration/ distribution, but diffused mosaicism occurred, leading to some cells in tissue affected by IC2 LOM	Likely occurred before migration/ distribution, leading to some cells in tissue affected by IC2 LOM, at similar levels to blood	Likely occurred before migration/ distribution, but diffused mosaicism occurred, leading to some cells in tissue affected by IC2 LOM, at varying levels to blood	Diffused mosaicism likely occurred during migration/ distribution, resulting in some cells in some tissues affected by IC2 LOM
Burden in tissue	Complete (c-LOM)	Partial (p-LOM)	Partial (p-LOM)	Partial (p-LOM)	Partial (p-LOM)
Degree of mosaicism	Nonmosaic early event with symmetric distribution	Nonmosaic early event with asymmetric distribution	Mosaic event with symmetric distribution	Mosaic event with asymmetric distribution	Mosaic later event with asymmetric distribution
Burden of affected cells	Constitutional c-LOM (concordant burden)	Constitutional c-LOM (discordant burden)	Constitutional p-LOM (concordant burden)	Constitutional p-LOM (discordant burden)	No constitutional Burden (discordant tissue burden)
	Consistent complete LOM demonstrated in samples (>95% of cells affected by LOM, c-LOM, p-LOM > IC2 LOM)	Consistent LOM demonstrated, with blood affected by high level of affected cells, and tissue affected by some affected cells	Consistent partial LOM demonstrated, with similar levels of affected cells between samples	Consistent partial LOM demonstrated, with different levels of affected cells between samples	Discordant LOM demonstrated, with only tissue having affected cells

variable mosaic distribution, with some tissues affected by IC2 LOM cells and some tissues not affected (Table 4). In the present study, four individuals were classified with a tissue-mosaic IC2 LOM event and the clinical phenotype ranged from classic BWSp to isolated lateralized overgrowth, which demonstrates the full clinical spectrum of BWSp and provides evidence for the phenotypic consequences of diffused mosaicism.

Burden of Affected Cells in IC2 LOM

A corollary mechanism to diffused epigenetic mosaicism is that all tissues do have some cells affected by IC2 LOM (albeit below the current testing detection level), but the burden of cells is not at the threshold to cause malformations or clinical issues. The consequential BWSp phenotype is likely related to the distribution of cells and the threshold of the tissue environment required to cause abnormalities. A correlation in the degree of IC2 LOM and severity of

BWSp phenotype was demonstrated in this study, which suggests that the molecular burden of IC2 LOM contributes to BWSp phenotype. We propose the "epigenetic burden theory" to complement the theory of diffused epigenetic mosaicism in BWSp. It appears that in individuals with BWSp due to IC2 LOM, the phenotypic presentation and clinical issues are dependent on the number of IC2 LOM affected cells in each tissue (consistent with the theory of diffused mosaicism) and that a threshold for the number of abnormal cells required to develop a phenotype may exist and furthermore be tissue-dependent. The distribution of cells and which tissues are affected are likely correlated with the embryologic developmental timing of different systems; however, this is challenging to study in affected individuals. Support for this phenomenon was recently presented by Sun et al. (2021) in a report of female twins who were concordant for the BWSp phenotype, but discordant for IC2 LOM in blood sample analysis. It was suggested that the twin with negative blood testing had cells affected by IC2 LOM during development that were distributed to the affected areas in her body such as tongue and intra-abdominal organs and that the presence of these cells was above the threshold that led to her phenotype (Sun et al. 2021).

Specific Phenotypic Consequences of the "Epigenetic Burden Theory"

Several features classified within the BWSp scoring system have common underlying affected systems, but varying degrees of affectedness. For example, AWD can be stratified as severe (omphalocele) or mild (umbilical hernia and/or diastasis recti), and hypoglycemia can be stratified as severe (HI) or transient. The severity of macroglossia has not yet been characterized in the BWSp population; however, it is accepted that tongue reduction (TR) surgery occurs in the context of severe macroglossia. In addition, individuals may not be affected at all by a feature, as demonstrated in our cohort: two individuals were not affected by macroglossia, two without AWDs, and five without any hypoglycemia, suggesting that the number of cells affected by IC2 LOM cells is below the burden to cause disease in the related tissue environments. The degree of LOM mosaicism appeared to be correlated with some of the common BWSp features, in which more severe phenotypic features will present in the context of high burden of IC2 LOM within organs and systems during embryonic and fetal development (Table 5). One clear example of this relationship was evident by the stratification of omphalocele with c-LOM, whereas minor AWD, or no AWD at all, appeared more common in partial or mosaic LOM.

There is no clear established mechanism leading to omphalocele; however, it is considered a result of failure of progression of normal embryonic development, and the embryonic dysplasia theory is among the most widely accepted (Khan et al. 2019). The embryonic dysplasia theory suggests that defects in the germinal disc occur extremely early in gestation, with manifestation occurring later in the development as malformations affecting the craniofacial region, body wall, and limbs (Khan et al. 2019). Several theories related to embryologic timing related to the development of omphalocele exist: some have been suggested that the development may begin before or around the third week of gestation, whereas others suggest that the development occurs between 8 and 12 wk of gestation (Khan et al. 2019). In three individuals in our study, the first prenatal sonographic evidence of omphalocele was detected at 12, 12 1/7, and 12 2/7 wk gestation, suggesting early abnormal development. All three individuals were affected by nonmosaic IC2 LOM (i.e., c-LOM detected in all samples), which suggests that the IC2 LOM cells were present when the abdominal wall began to form.

The Embryonic Dysplasia Theory

The embryonic dysplasia theory and associated malformations provide an interesting perspective to the BWSp phenotype. In addition to AWD and macroglossia, a variety of

Table 5. Description of Beckwith–Wiedemann spectrum (BWSp) phenotypic features, associated developmental systems with clinical correlation, and application of epigenetic burden theory

				Application of epigenetic burden theory	
Common BWSp features/ phenotypes	Embryonic/fetal developmental system(s) implicated	Clinical presentation types observed	Clinical correlation to (epi)genotype–phenotype	Observations from BWSp-IC2 LOM subgroup	Proposed application to other BWSp (epi) genotype groups[a]
Macroglossia	Tongue (craniofacial)	Isolated feature; or in constellation with other BWSp features	Associated with tissue mosaicism	Degree of IC2 LOM appears correlated with phenotype severity Severe LOM may indicate need for tongue reduction surgery	Associated with tissue mosaicism in pUPD and IC1 GOM[b,c]
Hypoglycemia or HI	Pancreas (organ systems)	May be presenting phenotype for subset of patients; or may represent one feature in constellation with others	Correlation appears to exist between degree of IC2 LOM (blood) and severity of hypoglycemia issues	Degree of IC2 LOM in pancreas is likely greater in patients affected by more severe degrees of hypoglycemia (i.e., HI) than transient forms	Hypoglycemia occurs in ~50% of all patients with BWSp; HI more common in IC2 LOM and pUPD[d,e]; pUPD associated with severe HI forms requiring pancreatectomy in context of tissue mosaicism[e,f]
Omphalocele; umbilical hernia; diastasis recti	Abdominal wall (gastrointestinal system) begins week 3 of gestational development[g]	Often the first detectable BWSp-related feature (evidenced at 12 wk within present study)	Correlation between degree of IC2 LOM and severity of AWD phenotype	More severe LOM (i.e., complete) appears correlated with omphalocele; partial appears correlated with minor defects (i.e., umbilical hernia and/or diastasis recti)	Omphalocele associated with CDKN1C mutations (IC2 region)[d]; minor AWD more common in pUPD or IC1 GOM (omphalocele is rare or less common)[d,e]
Lateralized overgrowth (LO) or asymmetry	Musculoskeletal (limbs) and/or craniofacial (face and/or tongue) organ(s)	May present as an isolated feature or in constellation with other common BWSp features	Associated with tissue mosaicism	No clear established relationship between IC2 LOM (blood) and LO phenotype; likely represents phenotype for tissue mosaicism and asymmetry of LOM burden between cell populations	Association with tissue mosaicism has been established[d–f]; phenotype appears common within MLIDs[d,h]

(LOM) Loss of methylation, (pUPD) paternal uniparental disomy, (GOM) gain of methylation, (HI) hyperinsulinism, (AWD) abdominal wall defect, (MLiDs) multilocus imprinting disturbances.

[a]Hypothesized influence derived from supporting literature evidence, with full references included within this article.

[b]Alders et al. (2014).

[c]Calvello et al. (2013).

[d]Brioude et al. (2018).

[e]Duffy et al. (2019).

[f]Kalish et al. (2016).

[g]Khan et al. (2019).

[h]Fontana et al. (2020).

craniofacial abnormalities have been reported (Maas et al. 2016), and limb asymmetry is also common, occurring with other BWSp features or as an isolated feature (Brioude et al. 2018; Duffy et al. 2019). If the embryonic dysplasia theory is considered in the context of BWSp, it is possible that the genes in the Chromosome 11p15 region may influence germinal disc development and the epigenetic alterations occur in temporal proximity to the establishment of specific cell lineages. It is likely that diffused epigenetic mosaicism occurs during the developmental process, and the affected cells are then asymmetrically distributed between the tissues. The resultant phenotype is dependent on which tissues have affected cells at levels above the threshold burden for that tissue, consistent with the later manifestation of malformations that can occur in the embryonic dysplasia theory (Khan et al. 2019). As demonstrated in the IC2 LOM subgroup, it appears that the threshold required for development of malformations may be different among tissues.

Mosaic levels of IC2 LOM were demonstrated between blood and tongue samples from the same individuals, providing direct evidence that different populations of LOM cells can exist in tissues. In the present study, we observed higher IC2 methylation levels in tongue compared to blood for most individuals (i.e., degree of affected cells in tongue is less than blood), which has been reported previously (Alders et al. 2014). Although it has been suggested that the IC2 region may be generally more methylated in tongue than blood (Alders et al. 2014), we observed four individuals affected by lower IC2 methylation levels detected in tongue compared to blood samples, which demonstrates that the tongue can be less methylated than blood. In two individuals in the present study, blood was negative for IC2 LOM, and the percentage of affected cells in the tongue sample was low but detectable, which suggests that the threshold for the macroglossia phenotype in BWSp may be lower compared to other tissues (e.g., a smaller number of affected LOM cells are needed to cause macroglossia, whereas a larger number of affected LOM cells are needed to cause omphalocele). The potential relationship of methylation levels in blood to the tongue remains unclear; however, these observations demonstrate the extent of epigenetic burden that can occur among tissues for each individual.

Summary

We have demonstrated that complete IC2 LOM levels detectable in blood, tongue, skin, and amniocytes are compatible with life (i.e., all cells affected by LOM); however, it is possible that a tissue-specific threshold exists in which the burden of IC2 LOM cells in some tissues may reach a level that comprises the environment required for the normal embryonic developmental process. It has been suggested that in individuals affected by BWSp, twinning occurred in all pregnancies, but the second fetus was resorbed early in the pregnancy (i.e., vanishing twin), resulting in a singleton (Bliek et al. 2009). Several theories can be applied to the embryologic developmental consequences of the IC2 LOM defect that provide support for this hypothesis. If the IC2 LOM defect occurs before the establishment of the germ layers, as we propose in the theory of diffused mosaicism, it is possible that early IC2 LOM events may result in germ layer defects that lead to fetal demise. If the embryonic dysplasia theory is accepted in the context of diffused mosaicism, it is possible that during fetal development, some tissues may receive populations of IC2 LOM cells at a burden that leads to severe malformations consistent with limb body wall complex (LBWC), which is lethal compared to other AWDs (Chikkannaiah et al. 2013). In the surviving fetus (or fetuses in the case of a multiple gestation pregnancy), the burden of the IC2 LOM cells in the affected tissues is compatible with life but can lead to variable manifestations, which are likely related to the distribution of affected cell populations. This concept of cell survival and compatibility may suggest the role of HSCs, which has previously been proposed within the context of IC2 LOM and BWS (Bliek et al. 2009; Sun et al. 2021). It is possible that HSCs may have some advantages over other cell types during diffused

mosaicism that contributes to the proposed environmental thresholds proposed in this study; further exploration may be warranted.

In summary, we provide evidence for the full molecular spectrum that can occur in the context of IC2 LOM, as well as the phenotypic spectrum that develops because of the IC2 LOM defect. These spectrums appear correlated, in that the severity of phenotype is the consequence of the number of cells affected by IC2 LOM in each tissue (i.e., a higher number of affected cells causes development of a more severe phenotype). Furthermore, we show that different tissues collected from the same individual can demonstrate varying levels of IC2 LOM, supporting the theory of diffused epigenetic mosaicism and providing indirect evidence that mosaic distribution of IC2 LOM occurs during embryogenesis. We suggest that the burden of affected cells in specific tissues drives the BWSp phenotype. Previously, the number of cells affected by IC1 GOM has been correlated with certain BWSp features, and it was suggested that a direct association between methylation percentage and severity of BWS exists (Calvello et al. 2013). It is likely that these theories extend to other (epi)genotype groups beyond those affected by IC2 LOM or IC1 GOM, and we have provided some thoughts about the proposed application within the BWSp population in Table 5. As we continue to characterize the clinical and molecular spectrum of BWS, it is important to recognize the mosaicism in each of the BWSp epigenotype subgroups to allow for characterization within each epigenotype in addition to between epigenotype subtypes. This will allow us to further improve diagnosis along with current and future management of these individuals.

METHODS

Participants and Samples

Eligible individuals included those with molecular testing performed at a single laboratory with a diagnosis of IC2 LOM detected in a blood sample with additional testing performed on any of the following tissues: (1) tongue sample, (2) skin biopsy sample, and/or (3) amniocentesis testing (prenatal sample). One individual with a skin biopsy testing result without blood analysis was also included. All individuals were enrolled in the BWS Registry, and they or their parents provided consent for medical record review, sample collection, and sample analysis. The mothers of individuals with prenatal testing were also enrolled in the BWS Registry and provided consent for review of medical records. Detailed methodology for the BWS Registry has been described (Duffy et al. 2019).

All samples were collected during routine clinical care. Tongue samples were collected from the leftover tissue following TR procedures. Skin biopsy samples were collected from the site of the surgical margin, and in one individual, the biopsy was collected directly from the larger leg affected by lateralized overgrowth during an outpatient clinical exam. Blood samples were collected as part of the individual's diagnostic evaluation, and amniocentesis samples were collected from the individual's mother as part of her prenatal care.

11p15.5 Methylation Analysis

All samples underwent 11p15.5 methylation analysis in a single laboratory, with detection methods for IC1 and IC2 as previously described (Baker et al. 2021a,b).

Data Analysis

BWSp Clinical Score

The BWSp clinical score was calculated based on the consensus criteria (Brioude et al. 2018) with two points assigned for each *cardinal feature* (four possible): macroglossia,

omphalocele, lateralized overgrowth, and/or hyperinsulinism (HI); and one point assigned for each *suggestive feature* (seven possible): large for gestational age (LGA), facial nevus simplex, ear creases/pits, transient hypoglycemia, minor AWD (umbilical hernia and/or diastasis recti), organomegaly (nephromegaly and/or hepatomegaly), and pregnancy findings (polyhydramnios and/or placentomegaly). Individuals with omphaloceles were not considered to be affected by minor AWD, although some did experience umbilical hernia after omphalocele repair. In this study, the maximum BWSp score possible was 13 points (four cardinal [two points each] + five suggestive). Of note, we did not evaluate or include points for tumors or pathology findings; however, these features are included in the BWSp clinical scoring criteria established by the international consensus.

Demographic and Clinical Characteristics

Demographic and clinical characteristics included individual sex, conception type (natural, in vitro fertilization [IVF], and/or intracytoplasmic sperm injection [ICSI]), multiple gestation status, preterm birth (<37 wk gestation), TR, and select BWSp features. BWSp phenotypes included macroglossia, lateralized overgrowth, overall presence of an AWD with stratifications (omphalocele or minor AWD), and overall presence of hypoglycemia with stratifications (HI or transient hypoglycemia).

IC2 LOM Subgroup Classifications

The following definitions were applied when evaluating the methylation results for each sample from individuals:

- Classification of IC2 LOM Molecular Type was considered for each individual as (a) *Constitutional IC2 LOM* (positive for IC2 LOM in blood and tissue sample) or (b) *Tissue Mosaic IC2 LOM* (positive for IC2 LOM in tissue sample, with negative blood analysis).
- Degree of IC2 LOM in each sample was categorized as (a) *c-LOM IC2* (complete LOM with <2.5% detected at IC2 in sample) or (b) *p-LOM IC2* (partial LOM with ≥2.5% detected at IC2 in sample).
- Mosaic distribution groups were defined as (a) *c-LOM IC2* (consistently identified in samples); (b) *p-LOM IC2* (consistently identified in samples); or (c) *mosaic LOM IC2* (discrepant methylation degrees between samples [i.e., c-LOM in blood, p-LOM in tissue]).

Statistical Analyses

All data were entered into an Excel database. Descriptive statistics were performed on all variables: continuous variables were summarized with mean and standard deviation (SD), and nominal/categorical variables were summarized with frequencies based on the distribution of affected individuals to those with available data for each variable. The relationship of BWSp clinical score to IC2 methylation percentage in blood was evaluated with Pearson correlation testing performed using SPSS (version 26); statistical significance was set at $P < 0.05$.

ADDITIONAL INFORMATION

Data Deposition and Access

The data that support the findings of this study have been provided in Supplemental Tables 1 and 2. Additional data may be available from the corresponding author upon reasonable request.

Ethics Statement

All participants were enrolled in the BWS Registry, which is approved by the Institutional Review Board at Children's Hospital of Philadelphia (IRB 13-010658). As part of enrollment in the BWS Registry, consent was obtained from the affected individual or legal guardian for collection of clinical information and samples. This work was performed in accordance with the ethical standards of the World Medical Society Declaration of Helsinki on ethical principles for medical research. No animals were used in this work.

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Author Contributions

K.A.D. and J.M.K. conceptualized and designed the study. E.R.H., J.M.K., and K.A.D. participated in aspects of clinical data collection and coordination of sample collection. A.G. oversaw the methylation analysis and interpretation of methylation results. K.A.D. performed the data collection and analysis and wrote the initial manuscript draft. J.M.K. and S.D.K. edited and revised the manuscript. All authors contributed to the manuscript draft and provided approval for the final version.

Competing Interest Statement

The authors have declared no competing interest.

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