

Epigenetic Alterations of *H19* and *LIT1* Distinguish Patients with Beckwith-Wiedemann Syndrome with Cancer and Birth Defects

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Beckwith-Wiedemann syndrome (BWS) is a congenital cancer-predisposition syndrome associated with embryonal cancers, macroglossia, macrosomia, ear pits or ear creases, and midline abdominal-wall defects. The most common constitutional abnormalities in BWS are epigenetic, involving abnormal methylation of either *H19* or *LIT1*, which encode untranslated RNAs on 11p15. We hypothesized that different epigenetic alterations would be associated with specific phenotypes in BWS. To test this hypothesis, we performed a case-cohort study, using the BWS Registry. The cohort consisted of 92 patients with BWS and molecular analysis of both *H19* and *LIT1*, and these patients showed the same frequency of clinical phenotypes as those patients in the Registry from whom biological samples were not available. The frequency of altered DNA methylation of *H19* in patients with cancer was significantly higher, 56% (9/16), than the frequency in patients without cancer, 17% (13/76; $P = .002$), and cancer was not associated with *LIT1* alterations. Furthermore, the frequency of altered DNA methylation of *LIT1* in patients with midline abdominal-wall defects and macrosomia was significantly higher, 65% (41/63) and 60% (46/77), respectively, than in patients without such defects, 34% (10/29) and 18% (2/11), respectively ($P = .012$ and $P = .02$, respectively). Additionally, paternal uniparental disomy (UPD) of 11p15 was associated with hemihypertrophy ($P = .003$), cancer ($P = .03$), and hypoglycemia ($P = .05$). These results define an epigenotype-phenotype relationship in BWS, in which aberrant methylation of *H19* and *LIT1* and UPD are strongly associated with cancer risk and specific birth defects.

Introduction

Beckwith-Wiedemann syndrome (BWS [MIM 130650]) is a model for the understanding of the role of epigenetics in cancer, since it involves frequent alterations in the imprinting and methylation of several genes on chromosomal band 11p15, including insulin-like growth factor-II (*IGF2* [MIM 147470]), *H19* (MIM 103280), *p57^{KIP2}* (or *CDKN1C* [MIM 600856]), *KvLQT1* (MIM 192500), and *LIT1* (MIM 604115). Genomic imprinting is a gene modification that typically involves DNA methylation but may include other factors as well and that leads to the relative silencing of a specific parental allele in somatic cells of the offspring. The phenotypic features of BWS are macrosomia, neonatal hypoglycemia, midline abdominal defects (e.g., omphalocele, diastasis recti, and umbilical hernia), macroglossia, and ear pits or ear creases, as well as an increased incidence of

embryonal cancers in infants and young children. The most frequent cancer is Wilms tumor, occurring in 11% of children with BWS before 4 years of age, followed in frequency by other embryonal tumors, including hepatoblastoma and neuroblastoma (DeBaun and Tucker 1998). Other cancers occurring with lower frequency in this population include rhabdomyosarcoma and adrenocortical carcinoma.

The epigenetics of BWS is complex. Six genetic or epigenetic alterations in patients with BWS have been described:

1. Loss of imprinting (LOI) of *IGF2* in embryonal tumors and in the normal cells of patients with BWS, which, in BWS, involves activation of the normally silent maternally inherited allele of *IGF2* (Ogawa et al. 1993; Rainier et al. 1993; Weksberg et al. 1993). Most investigators report a frequency of ~10%–15% (Elias et al. 1998; Lee et al. 1999), but one study suggests a higher frequency (Joyce et al. 1997).

2. Abnormal methylation of the normally unmethylated allele of a differentially methylated region (DMR) upstream from *H19* (Steenman et al. 1994; Reik et al. 1995). The *H19* DMR serves as a site for the binding of the insulator protein CTCF, and it inhibits access of

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the maternal allele of *IGF2* to a shared enhancer downstream from *H19* (reviewed by Feinberg [2001]). In the case of Wilms tumor, LOI of *IGF2* is coupled to abnormal *H19* methylation (Moulton et al. 1994; Steenman et al. 1994). In the case of BWS, there may also be an independent mechanism for regulating *IGF2* in these patients (Joyce et al. 1997). The frequency of this alteration is 15% (reviewed by Elias et al. [1998]).

3. Germline chromosomal rearrangements. We showed that balanced rearrangements interrupt the *KvLQT1* gene (Lee et al. 1997b), and there are also rare unbalanced duplications (reviewed by Elias et al. [1998]).

4. Mutations of the *p57^{KIP2}* gene, which are rare (Lee et al. 1997a; O'Keefe et al. 1997).

5. LOI of *LIT1*, shown directly by us (Lee et al. 1999) and indirectly by others (Smilinich et al. 1999). *LIT1* is an antisense transcript that is normally expressed from the paternal allele and lies within the *KvLQT1* gene (Lee et al. 1999; Smilinich et al. 1999), and LOI of *LIT1* is completely linked to abnormal methylation of a DMR upstream from the gene (Lee et al. 1999). This is the most frequent abnormality in BWS, accounting for 40%–50% of patients.

6. Paternal uniparental disomy (UPD) of 11p15, occurring in ~10% of patients (reviewed by Elias et al. [1998]).

We hypothesized that there may be a relationship between cancer risk and LOI of *IGF2* and/or aberrant methylation of the *H19* DMR, because we and others have described both defects in the general population, in the same type of embryonal cancers found in children with BWS (Ogawa et al. 1993; Rainier et al. 1993; Steenman et al. 1994). In contrast, we and others have found no alterations in *LIT1*, *p57^{KIP2}*, or *KvLQT1* in embryonal tumors (Reid et al. 1996; Mitsuya et al. 1999; M. P. Lee and A. P. Feinberg, unpublished data), suggesting specificity of the *H19/IGF2* subdomain in embryonal tumors. In preliminary data, we described the association between abnormal *H19* methylation and increased cancer risk in BWS (Niemitz et al. 2001). In addition, Blik et al. (2001) documented an association between aberrant *H19* methylation and cancer; however, in that study, it was unclear whether cancer was specific for abnormal *H19* methylation or UPD, as there were only two cancer patients with abnormal *H19* methylation in the absence of UPD. Additionally, Engel et al. (2000) described a 69% (20 of 29) frequency of midline abdominal-wall defects in patients with abnormal methylation of *LIT1*, but they did not compare this frequency to all patients with BWS without abnormal *LIT1* methylation, so it cannot be determined whether this frequency is elevated. Because of small numbers of patients in both studies, discrimination of phenotypic association for UPD versus the individual affected genes could

not be done. Both studies most likely had strong ascertainment bias, since children with either cancer or midline abdominal-wall defects who required surgery were probably referred to tertiary care centers. The current study addressed a large non-tertiary-care-center-based registry of children with BWS, and it represents the first report of BWS to examine multiple phenotypes associated with a given epigenotype, allowing the definition of epigenotype-phenotype relationships.

Patients and Methods

Patients

We established the BWS Registry in 1994 at the Genetic Epidemiology Branch of the National Cancer Institute to understand the natural history of this syndrome and to determine whether genotype-phenotype relationships exist. For those families who agreed to participate, a comprehensive questionnaire was completed, and informed consent and medical-information-release documents were obtained, as approved by the respective institutional review boards. For the purposes of this study, we defined a patient as having BWS if a clinical diagnosis of BWS had been made by a physician and the patient had at least two of the five most common features associated with BWS (Pettenati et al. 1986): (1) macroglossia, (2) birth weight and length >90th percentile, (3) hypoglycemia in the first month of life, (4) ear creases or ear pits, and (5) midline abdominal-wall defects (omphalocele, diastasis recti, and umbilical hernia). All positive phenotypic features were diagnosed by a physician and were documented in the questionnaire. The local physician defined idiopathic hemihypertrophy. In four patients, birth weight and length were unknown, and, thus, macrosomia could not be scored in those four patients. If a cancer was reported, the diagnosis was confirmed by review of the final pathology report and medical records. Among the patients studied, there were 16 embryonal tumors, including 13 Wilms tumors, 2 hepatoblastomas, and 1 rhabdomyosarcoma. The genotypes of all patients were not known prior to entering the registry. All molecular analyses were performed blinded for knowledge of the phenotype.

Isolation of DNA

Genomic DNA was prepared from peripheral-blood lymphocytes (2/3 of patients) or cultured skin fibroblasts (1/3 of patients) by standard proteinase K digestion and phenol extraction (Cui et al. 1998). This material is suitable for methylation assays involving DNA.

Analysis of *H19* Methylation

A 1.8-kb *PstI* fragment of the *H19* gene (nucleotides 1–1803 of the human *H19* probe [GenBank accession

number M32053]) was analyzed for DNA methylation by digestion with *Pst*I and *Sma*I. Ten micrograms of DNA were digested at 25°C overnight with 40 U of *Sma*I, followed by an additional incubation at 37°C for 4 h with 40 U of *Pst*I. The digested DNA was electrophoresed on a 1% agarose gel, was transferred to Hybond-N⁺ (Amersham), and was hybridized with the 1-kb *Pst*I + *Sma*I fragment isolated from an *H19* genomic clone and oligolabeled with α -[³²P]-dATP. Signals were quantified using a PhosphorImager (Molecular Dynamics). Abnormal methylation in lymphocytes was defined as a methylation index of >0.63 (mean + 2 SD of 15 normal individuals). DNA samples were available from blood or fibroblasts but not both from any given patient. Fibroblasts from normal individuals ($n = 5$) showed a methylation index of ~0.33. All of the BWS fibroblast samples showed a methylation index of ~0.33 (scored as normal) or ~0.66 (scored as abnormal). There was no difference in the frequency of increased methylation when comparing BWS samples from lymphocytes or BWS samples from fibroblasts. Furthermore, the association between abnormal *H19* methylation and cancer was unchanged when the fibroblast samples were excluded.

Analysis of *LIT1* Methylation

Ten micrograms of DNA were digested with *Bam*HI and *Not*I at 37°C overnight. The digested DNA was electrophoresed on a 1% agarose gel, was transferred to Hybond-N⁺ (Amersham), and was hybridized with an expressed-sequence-tag probe (GenBank accession number AA155639) oligolabeled with α -[³²P]-dATP. Signals were quantified using a PhosphorImager (Molecular Dynamics). Abnormal methylation was defined as a methylation index of <0.39 (mean – 2 SD of 15 normal individuals). The methylation index of *LIT1* was unchanged in cultured skin fibroblasts.

Analysis of UPD

Patients who were determined to have epigenetic alterations at both BWS loci tested (i.e., to have hypermethylation of *H19* and hypomethylation of *LIT1*) were evaluated for paternal UPD of 11p15.5. Patient and maternal DNA samples were genotyped with a panel of three microsatellite markers: D11S1318, D11S988, and D11S922. PCR primer sequences were obtained from the Genome Database. PCR was performed as recommended by the Genome Database by use of FAM-labeled primer pairs. PCR products were then analyzed by electrophoresis on a model 377 automated fluorescent DNA sequencer (Applied Biosystems). The raw data were analyzed using Genescan software (Applied Biosystems). The peak data generated by Genescan were then analyzed using the Genotyper program (Applied Biosys-

tems). Patients in whose DNA the Genescan software indicated that the maternal allele(s) was absent were considered to have UPD; however, examination of the chromatograms for those patients revealed a small peak (<20% of the scored allele), corresponding to one of the maternal alleles and thereby indicating mosaicism for UPD for each patient.

Statistical Analysis

A χ^2 statistic and Fisher's exact test were used to determine whether specific epigenotypes (aberrant methylation of either *H19* or *LIT1* or UPD of 11p15) were associated with the presence of specific phenotypes in children with BWS. When the expected value in a given cell was <5, Fisher's exact test was used (Glantz 1992). A *P* value <.05 was considered statistically significant. In addition, the odds ratio for cancer was calculated and was considered to be significantly increased if it was >1.0 and if the 95% confidence intervals (95% CIs) did not include 1.0.

Results

Comparison of Patients with BWS, with and without Molecular Analysis

A total of 279 children have met the criteria to be included in the BWS Registry, of whom material could be obtained from 92 for analysis of both *LIT1* and *H19* methylation and UPD. The average age of patients who provided biological specimens was 5.6 years, and the age range was 1 mo–30 years. Of the patients, 52% were male, and, of the parents, 87% identified themselves as white, 2% identified themselves as African American, 7% identified themselves as Hispanic, 2% identified themselves as Asian, and 2% identified themselves as Native American. No significant phenotypic differences were identified between patients who were molecularly analyzed and those who were not, including frequency of cancer, macroglossia, hypoglycemia, abdominal-wall defects, macrosomia, ear pits or creases, and hemihypertrophy (table 1). These findings indicate that the cohort of children with biological specimens in the BWS Registry were representative of the entire BWS Registry.

Frequency of Genotypes

The most frequent molecular alteration observed in children with BWS was altered *LIT1* methylation with normal *H19* methylation (42% [39/92 patients]). The frequency of altered *H19* methylation with normal *LIT1* methylation was 11% (10/92 patients). Twelve patients (13%) exhibited altered methylation of both *H19* and *LIT1*. We expected that these patients would show paternal UPD, since, in UPD, both maternal copies are

Table 1
Comparison of Patients with BWS, with and without Molecular Analysis

PHENOTYPE	% WITH PHENOTYPE		P ^a
	With Molecular Analysis (n = 92)	Without Molecular Analysis (n = 187)	
Macrosomia	88	53	.25
Ear grooves/pits	63	71	.17
Abdominal-wall defects	68	76	.15
Hypoglycemia	53	52	.83
Macroglossia	97	92	.16
Hemihypertrophy	36	32	.47
Embryonal cancer	16	19	.08

^a Calculated by χ^2 analysis.

replaced with a paternal copy that alters the methylation pattern of both genes. Unexpectedly, of these 12 patients, 9 showed UPD, and the remaining 3 exhibited altered methylation of both *H19* and *LIT1* in the absence of UPD. These patients therefore had an imprinting disturbance extending throughout the 11p15 imprinted-gene domain, which has not been described elsewhere.

Association of Cancer with Altered *H19* Methylation

Abnormal *H19* methylation was associated with an elevated risk for embryonal cancer (table 2). The frequency of altered *H19* methylation in patients with embryonal cancer was significantly greater than that in patients without cancer—56% (9/16) versus 17% (13/76), respectively ($P = .002$). The odds ratio for cancer in children with altered *H19* methylation was 6.2 (95%CI 1.7–19.8). In contrast, altered *LIT1* methylation was not associated with an elevated risk for embryonal cancer (table 2). We also determined whether the increased frequency of altered *H19* methylation in patients with cancer was specific for *H19* or was simply due to UPD or an imprinting disturbance extending throughout the 11p15 imprinted-gene domain. To address this possibility, we excluded patients with UPD ($n = 9$) or with aberrant methylation of both *H19* and *LIT1* ($n = 3$) from the analysis; with the exclusion of these 12 patients, the frequency of altered *H19* methylation among patients with embryonal cancer was still significantly higher than that in patients without cancer—36% (4/11) versus 9% (6/69), respectively ($P = .01$). The odds ratio for cancer in children with altered *H19* methylation—6.0 (95%CI 1.3–26.5)—also did not change significantly when these 12 patients were excluded, providing strong evidence that the risk of cancer was specific for abnormal *H19* methylation and was not simply due to UPD. Therefore, abnormal *H19* methylation appears to be specifically associated with increased cancer risk in BWS.

Association of Macrosomia and Midline Abdominal-Wall Defects with Altered Methylation of *LIT1*

We examined the association between molecular abnormalities in BWS and macrosomia, hypoglycemia, macroglossia, ear pits or ear creases, hemihypertrophy, and midline abdominal-wall defects. The frequency of altered *LIT1* methylation in patients with macrosomia was significantly higher than that in patients without macrosomia—60% (46/77) versus 18% (2/11), respectively ($P = .02$; table 2). Similarly, the frequency of altered *LIT1* methylation in patients with midline abdominal-wall defects was significantly higher than that in patients without such defects—65% (41/63) versus 34% (10/29), respectively ($P = .012$; table 2). For both macrosomia and midline abdominal-wall defects, we also determined whether the increased frequency of altered *LIT1* methylation was simply due to UPD or an imprinting disturbance extending throughout the 11p15 imprinted-gene domain. To address this possibility, we excluded patients with either UPD or aberrant methylation of both *H19* and *LIT1* from the analysis of macrosomia ($n = 11$) and midline abdominal-wall defects ($n = 12$). Excluding these patients, the frequency of altered *LIT1* methylation among patients with macrosomia was still significantly higher than that in patients without macrosomia—53% (35/66) versus 18% (2/11), respectively ($P = .03$). Similarly, the frequency of altered *LIT1* methylation among patients with midline abdominal-wall defects remained significantly higher than that in patients without such defects—60% (33/55) versus 24% (6/25), respectively ($P = .003$). Thus, the association between macrosomia and midline abdominal-wall defects was specific for abnormal *LIT1* methylation and was not simply due to UPD. Therefore, abnormal *LIT1* methylation appears to be specifically associated with macrosomia and midline abdominal-wall defects in BWS.

Association of Hemihypertrophy and Hypoglycemia with Altered Methylation of Both *LIT1* and *H19*

We determined whether the 12 patients with aberrant methylation of both *H19* and *LIT1* (including the 9 patients with UPD) uniquely differed in phenotype in some way—that is, we determined whether there were any features specifically associated with such a domain-wide alteration that were not found in association with *H19* or *LIT1* alone. We found that hemihypertrophy and hypoglycemia occurred significantly more frequently in these patients. The frequency of hemihypertrophy among patients with UPD or aberrant methylation of both *H19* and *LIT1* was significantly higher than that in patients without such defects—30% (10/33) versus 4% (2/49), respectively ($P = .003$). Similarly, the frequency of hypoglycemia was 20% (10/49) and 5%

Table 2
Significant Relationships between Phenotype and Molecular Abnormalities in BWS

PHENOTYPE	ABNORMAL <i>H19</i>		ABNORMAL <i>LIT1</i>		UPD ^a	
	Frequency	<i>P</i>	Frequency	<i>P</i>	Frequency	<i>P</i>
Cancer:						
Present	9/16 (56%)	.002 ^b	6/16 (38%)	.19	5/16 (31%)	.03 ^b
Absent	13/76 (17%)		45/76 (59%)		7/76 (9%)	
Macrosomia:						
Present	18/77 (23%)	1.0 ^b	46/77 (60%)	.02	11/77 (14%)	.3 ^b
Absent	2/11 (18%)		2/11 (18%)		0/11 (0%)	
Hypoglycemia:						
Present	14/49 (29%)	.4	28/49 (57%)	.9	10/49 (20%)	.05 ^b
Absent	8/43 (19%)		23/43 (53%)		2/43 (5%)	
Hemihypertrophy:						
Present	12/33 (36%)	.07 ^c	19/33 (58%)	.9	10/33 (30%)	.003 ^b
Absent	10/59 (17%)		32/59 (54%)		2/49 (4%)	
Midline defects:						
Present	14/63 (22%)	.76	41/63 (65%)	.012	8/63 (13%)	1.0 ^b
Absent	8/29 (28%)		10/29 (34%)		4/29 (14%)	

^a Plus cases of abnormal methylation of both *H19* and *LIT1*.

^b Determined by Fisher's exact test.

^c *P* = .5 if patients with UPD are excluded; thus, there is no independent association with *H19* methylation.

(2/43), respectively (*P* = .05). Thus, in BWS, abnormal methylation of both *H19* and *LIT1* appears to be specifically associated with hemihypertrophy and hypoglycemia. As expected, in these 12 patients, there was also a significantly increased frequency of cancer (table 2), since cancer is associated with abnormal methylation of only *H19* (table 2). Somewhat surprisingly, that was not the case for macrosomia or midline abdominal-wall defects, which showed no significant increase in frequency in these 12 patients, even though these abnormalities are associated with abnormal methylation of only *LIT1* (table 2).

Discussion

BWS is a model for the study of epigenetics in human disease, since it involves multiple imprinted genes, as well as both increased risk of cancer and a wide variety of birth defects. BWS offers a unique opportunity to understand the relationship between epigenotype and phenotype, because of its genetic heterogeneity and substantial variability in expressivity of the phenotype. The large number of patients in this study and their complete phenotypic assessment allowed for subgroup analysis to assess specific epigenotype-phenotype relationships. We focused this investigation on epigenetic changes in this disorder—specifically, altered methylations of *H19* and *LIT1*, which account for most of the known alterations in these patients. There were three significant epigenotype-phenotype associations revealed by this analysis: (1) cancer is significantly associated with altered methylation of *H19*; (2) macrosomia and midline abdominal-

wall defects are significantly associated with altered *LIT1* methylation; and (3) hypoglycemia and hemihypertrophy are significantly associated with UPD or altered methylation of both *LIT1* and *H19*.

The association, in BWS, between paternal UPD of 11p15 and cancer was initially suggested by Henry et al. (1993), in a small case series in which three of six patients with UPD had cancer. Similarly, because of the small number of patients with cancer (*n* = 7), Blik et al. (2001) could not perform a statistical analysis distinguishing the risk of cancer with UPD from the risk of cancer with abnormal *H19* methylation. Here, we show that this risk is specific for abnormal *H19* methylation.

Given that the epigenetic alteration associated with cancer risk in BWS is altered methylation of the *H19* DMR, what might be the mechanism for cancer in these patients? The only known function of *H19* is the regulation of *IGF2* expression by the *H19* DMR, since knockout of the *H19* RNA itself has no effect in mice (Schmidt et al. 1999). We and others previously showed that sporadically occurring embryonal tumors (i.e., those not occurring in the context of BWS) undergo LOI of *IGF2* (Ogawa et al. 1993; Rainier et al. 1993). We found that LOI of *IGF2*, which involves activation of the normally silent maternal allele, is often coupled, in these tumors, to abnormal methylation of the maternal allele of the *H19* DMR (Moulton et al. 1994; Steenman et al. 1994). This methylation appears to inhibit binding of CTCF or of other insulator proteins to the normally unmethylated maternal *H19* DMR, thus allowing access by a shared enhancer to the *IGF2* promoter and thereby causing biallelic expression of *IGF2*

(reviewed by Feinberg [2001]). We hypothesized that a similar mechanism may be at work in patients with constitutional epigenetic alterations (i.e., in patients with BWS). Our study supports a role for the *H19* DMR in the pathogenesis of human cancer. The human *H19* DMR extends from the promoter to 6 kb upstream from *H19*, and the region examined here is the same (promoter region) as that identified earlier by us and others as showing abnormal methylation in Wilms tumor (Moulton et al. 1994; Steenman et al. 1994). We have found that altered methylation in Wilms tumor also includes CTCF-binding sites 5 kb upstream and within the DMR (Cui et al. 2001), and it will be of interest in further studies to map these sites precisely in BWS.

IGF2 imprinting could not be directly analyzed in most patients, since it would require examination of expressing tissues as well as informativeness for the requisite polymorphic markers to distinguish individual alleles. One problem with examining *IGF2* in cultured fibroblasts is that we have found *IGF2* to undergo loss of imprinting in cultured normal fibroblasts (E. L. Niemitz and A. P. Feinberg, unpublished data), which may explain the apparent discordance of *IGF2* imprinting and *H19* methylation reported by Joyce et al. (1997). In the study by Blik et al. (2001), the source of DNA was not given. We also found that *H19* methylation, but not *LIT1* methylation, is reduced in cultured skin fibroblasts; however, in the present study, there was no difference in the frequency of increased *H19* methylation among samples from patients with BWS derived from blood or from cultured skin fibroblasts, and thus altered imprinting of *IGF2* may be methylation independent—at least at the *H19* DMR. Note that the source of DNA did not affect the results, since the association between increased *H19* methylation and cancer was seen even if the patients with fibroblast samples were excluded.

Given that, in BWS, the epigenetic alteration associated with midline abdominal-wall defects and macrosomia is altered *LIT1* methylation, what is the mechanism for such congenital malformations in these patients? The function of *LIT1* is unknown, although we have previously hypothesized that abnormal hypomethylation of *LIT1* might cause the silencing of the nearby *p57^{KIP2}* gene, by a mechanism similar to the silencing of *IGF2* by the *H19* DMR (Lee et al. 1999). In support of this hypothesis, *p57^{KIP2}* is mutated in a small fraction of patients with BWS (Lee et al. 1997a; O'Keefe et al. 1997). Furthermore, knockout mice lacking a functional *p57^{KIP2}* gene show similar midline abdominal-wall defects (Zhang et al. 1997). As with *IGF2*, direct examination of *p57^{KIP2}* imprinting would require analysis of expressing tissues, such as kidney and tongue, which are not readily available in a large cohort of patients.

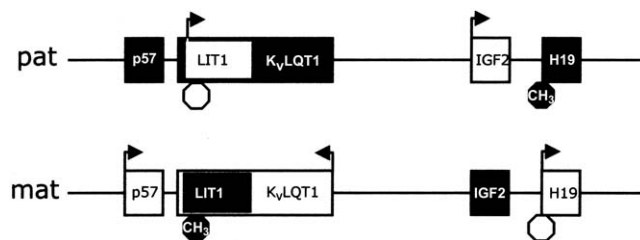


Figure 1 One-megabase region of chromosome 11p15.5 containing two imprinted-gene subdomains. The more centromeric subdomain includes *p57^{KIP2}*, *LIT1*, and *KvLQT1*, and the more telomeric subdomain includes *IGF2* and *H19*. Unblackened boxes represent transcriptionally active genes, and arrows indicate the transcriptional orientation; octagons represent the DMRs analyzed here, and blackened octagons indicate methylation. Paternal chromosome is designated by “pat”; maternal chromosome is designated by “mat.” (Not all genes are listed.)

These observations fit very well a two-domain hypothesis for BWS we have proposed elsewhere (Lee et al. 1999). By this model, 11p15 contains two separate imprinted subdomains (fig. 1). The more telomeric subdomain includes *IGF2* and *H19*, and the more centromeric subdomain includes *p57^{KIP2}*, *LIT1*, and *KvLQT1*. BWS arises by the disrupted imprinting of either subdomain. The likely target gene for BWS is *IGF2* in the more telomeric subdomain and *p57^{KIP2}* in the more centromeric subdomain. By this model, a DMR lies between a given BWS target gene and its enhancer, and abnormal hypermethylation permits access of this enhancer to the maternal allele of *IGF2* on the maternal allele, while abnormal hypomethylation inhibits access of this enhancer to the maternal allele of *p57^{KIP2}*. The chromosomal rearrangements in *KvLQT1* would have the same effect (Lee et al. 1997b). Support for our model comes from a comparative genomic analysis showing a similar organization in the mouse (Onyango et al. 2001) and from a study indicating that the *p57^{KIP2}* enhancer is in the location our model predicts, although the enhancer itself was not identified in that study (Cleary et al. 2001). The data in the current study would suggest that the more centromeric subdomain is involved primarily in growth regulation and that the more telomeric subdomain is involved primarily in tumor suppression.

An additional conclusion of this study is that hypoglycemia and hemihypertrophy are associated specifically with UPD or with methylation abnormalities affecting both *H19* and *LIT1*. We address each of these separately:

1. Hemihypertrophy. This is an asymmetric enlargement of one half of the body or a portion thereof. We suggest that hemihypertrophy in BWS is simply the mosaic form of macrosomia, which was associated with

altered methylation of *LIT1*. To date, all of the patients with UPD reported—including those studied here—are mosaic. We predict that there is an asymmetric spatial distribution of UPD on the two sides of the body in these patients.

2. Hypoglycemia. The association between hypoglycemia and UPD (or abnormal methylation of both genes) and the lack of association between hypoglycemia and either methylation of only *H19* or only *LIT1* suggest that another gene on 11p15 may contribute to this phenotype. Such a gene would also undergo paternal duplication in patients with UPD. What gene could this be? There are several additional imprinted genes on 11p15 (reviewed by Feinberg [1999]). One intriguing possibility is that the insulin gene (*INS* [MIM 176730]), although not generally thought to be imprinted, may show imprinting in the yolk sac (Moore et al. 2001). It will be of interest to examine the imprinting of the insulin gene in the developing pancreas.

As with any case-cohort study, our analysis is associated with several limitations. We did not obtain biological samples from all patients in the BWS Registry; however, the frequency of cancer and other phenotypic features did not differ significantly between those with and without molecular analysis. Another limitation is that we were not able to assess the relationship between phenotype and *p57^{KIP2}* mutations or *KvLQT1* rearrangements, because of the low frequency of these alterations; in our previous work and work by others, the frequency of *p57^{KIP2}* mutations is 5% (Lee et al. 1997a; O'Keefe et al. 1997), and the frequency of *KvLQT1* rearrangements is <1% (reviewed by Elias et al. [1998]). The focus of the present study has been epigenetic alterations, rather than mutations.

Finally, we have here reported the first examples of abnormal methylation that affects both *LIT1* and *H19*, in the absence of UPD. These results suggest that these three patients exhibit disrupted methylation throughout the 11p15 imprinted-gene domain. We postulate that there is a hierarchical epigenetic organization of this domain, with some patients showing epigenetic disruption including that of *LIT1*, with others showing epigenetic disruption including that of *H19*, and with a final group showing epigenetic disruption affecting the domain as a whole. Such patients may have “imprinting center” mutations analogous to those seen in some patients with Prader-Willi syndrome (MIM 176270) or Angelman syndrome (MIM 105830) with microdeletions (Buiting et al. 2000).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human *H19* probe [accession number M32053] and human *LIT1* probe [accession number AA155639])

Genome Database, The, <http://gdbwww.gdb.org/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for BWS [MIM 130650], *IGF2* [MIM 147470], *H19* [MIM 103280], *p57^{KIP2}* [MIM 600856], *KvLQT1* [MIM 192500], and *LIT1* [MIM 604115])

References

- Blik J, Maas SM, Ruijter JM, Hennekam RCM, Alders M, Westerveld A, Mannens MMAM (2001) Increased tumour risk for BWS patients correlates with aberrant *H19* and not *KCNQ1OT1* methylation: occurrence of *KCNQ1OT1* hypomethylation in familial cases of BWS. *Hum Mol Genet* 10:467–476
- Buiting K, Farber C, Kroisel P, Wagner K, Brueton L, Robertson ME, Lich C, Horsthemke B (2000) Imprinting centre deletions in two PWS families: implications for diagnostic testing and genetic counseling. *Clin Genet* 58:284–290
- Cleary MA, van Raamsdonk CD, Levorse J, Zheng B, Bradley A, Tilghman SM (2001) Disruption of an imprinted gene cluster by a targeted chromosomal translocation in mice. *Nat Genet* 29:78–82
- Cui H, Horon IL, Ohlsson R, Hamilton SR, Feinberg AP (1998) Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nat Med* 4:1276–1280
- Cui H, Niemitz EL, Ravenel JD, Onyango P, Brandenburg SA, Lobanenkova VV, Feinberg AP (2001) Loss of imprinting of *insulin-like growth factor-II* in Wilms' tumor commonly involves altered methylation but not mutations of *CTCF* or its binding site. *Cancer Res* 61:4947–4950
- DeBaun MR, Tucker MA (1998) Risk of cancer during the first four years of life in children from the Beckwith-Wiedemann Syndrome Registry. *J Pediatr* 132:398–400
- Elias ER, DeBaun MR, Feinberg AP (1998) Beckwith-Wiedemann syndrome. In: Jameson JL (ed) *Principles of molecular medicine*. Humana, Totowa, New Jersey, pp 1047–1052
- Engel JR, Smallwood A, Harper A, Higgins MJ, Oshimura M, Reik W, Schofield PN, Maher ER (2000) Epigenotype-phenotype correlations in Beckwith-Wiedemann syndrome. *J Med Genet* 37:921–926
- Feinberg AP (1999) Imprinting of a genomic domain of 11p15 and loss of imprinting in cancer: an introduction. *Cancer Res* 59 Suppl:1743s–1746s
- Feinberg AP (2001) Cancer epigenetics takes center stage. *Proc Natl Acad Sci USA* 98:392–394
- Glantz S (1992) *Primer of biostatistics: the program*. McGraw-Hill, New York

- Henry I, Puech A, Riesewijk A, Ahnine L, Mannens M, Beldjord C, Bitoun P, Tournade MI, Junien C (1993) Somatic mosaicism for partial paternal isodisomy in Wiedemann-Beckwith fertilization event. *Eur J Hum Genet* 1:19–29
- Joyce JA, Lam WK, Catchpoole DJ, Jenks P, Reik W, Maher ER, Schofield PN (1997) Imprinting of *IGF2* and *H19*: lack of reciprocity in sporadic Beckwith-Wiedemann syndrome. *Hum Mol Genet* 6:1543–1548
- Lee MP, DeBaun MR, Mitsuya K, Galonek HL, Brandenburg S, Oshimura M, Feinberg AP (1999) Loss of imprinting of a paternally expressed transcript, with antisense orientation to *KvLQT1*, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc Natl Acad Sci USA* 96:5203–5208
- Lee MP, DeBaun M, Richard BA, Elledge SJ, Feinberg AP (1997a) Low frequency of *p57^{KIP2}* mutation in Beckwith-Wiedemann syndrome. *Am J Hum Genet* 61:304–309
- Lee MP, Hu RJ, Johnson LA, Feinberg AP (1997b) Human *KvLQT1* gene shows tissue-specific imprinting and encompasses Beckwith-Wiedemann syndrome chromosomal rearrangements. *Nat Genet* 15:181–185
- Mitsuya K, Meguro M, Lee MP, Katoh M, Schulz TC, Kugoh H, Yoshida MA, Niikawa N, Feinberg AP, Oshimura M (1999) *LIT1*, an imprinted antisense RNA in the human *KvLQT1* locus identified by screening for differentially expressed transcripts using monochromosomal hybrids. *Hum Mol Genet* 8:1209–1217
- Moore GE, Abu-Amero SN, Bell G, Wakeling EL, Kingsnorth A, Stanier P, Jauniaux E, Bennett ST (2001) Evidence that insulin is imprinted in the human yolk sac. *Diabetes* 50:199–203
- Moulton T, Crenshaw T, Hao Y, Moosikasuwana J, Lin N, Dembitzer F, Hensle T, Weiss L, McMorrow L, Loew T (1994) Epigenetic lesions at the *H19* locus in Wilms' tumour patients. *Nat Genet* 7:440–447
- Niemitz EL, Feinberg AP, DeBaun MR (2001) Specific association of altered DNA methylation of the *H19* gene with cancer risk in Beckwith-Wiedemann syndrome. In: *Proceedings of the American Association of Cancer Research*, Vol 42. p 3796
- Ogawa O, Eccles MR, Szeto J, McNoe LA, Yun K, Maw MA, Smith PJ, Reeve AE (1993) Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* 362:749–751
- O'Keefe D, Dao D, Zhao L, Sanderson R, Warburton D, Weiss L, Anyane-Yeboah K, Tycko B (1997) Coding mutations in *p57^{KIP2}* are present in some cases of Beckwith-Wiedemann syndrome but are rare or absent in Wilms tumors. *Am J Hum Genet* 61:295–303
- Onyango P, Miller W, Lehoczy J, Leung CT, Birren B, Wheelan S, Dewar K, Feinberg AP (2001) Sequence and comparative analysis of the mouse 1-megabase region orthologous to the human 11p15 imprinted domain. *Genome Res* 10:1697–1710
- Pettenati MJ, Haines JL, Higgins RR, Wappner RS, Palmer CG, Weaver DD (1986) Wiedemann-Beckwith syndrome: presentation of clinical and cytogenetic data on 22 new cases and review of the literature. *Hum Genet* 74:143–154
- Rainier S, Johnson LA, Dobry CJ, Ping AJ, Grundy PE, Feinberg AP (1993) Relaxation of imprinted genes in human cancer. *Nature* 362:747–749
- Reid LH, Crider-Miller SJ, West A, Lee MH, Massague J, Weissman BE (1996) Genomic organization of the human *p57^{KIP2}* gene and its analysis in the G401 Wilms' tumor assay. *Cancer Res* 56:1214–1218
- Reik W, Brown KW, Schneid H, Le Bouc Y, Bickmore W, Maher ER (1995) Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by altered imprinting pattern in the *IGF2-H19* domain. *Hum Mol Genet* 4:2379–2385
- Schmidt JV, LeVorse JM, Tilghman SM (1999) Enhancer competition between *H19* and *Igf2* does not mediate their imprinting. *Proc Natl Acad Sci USA* 96:9733–9738
- Smilnich NJ, Day CD, Fitzpatrick GV, Caldwell GM, Lossie AC, Cooper PR, Smallwood AC, et al (1999) A maternally methylated CpG island in *KvLQT1* is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *Proc Natl Acad Sci USA* 96:8064–8069
- Steenman M, Rainier S, Dobry CJ, Grundy P, Horon IL, Feinberg AP (1994) Loss of imprinting of *IGF2* is linked to reduced expression and abnormal methylation of *H19* in Wilms' tumor. *Nat Genet* 7:433–439
- Weksberg R, Shen DR, Fei YL, Song QL, Squire J (1993) Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat Genet* 5:143–150
- Zhang P, Liegeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinto RA, Elledge SJ (1997) Altered cell differentiation and proliferation in mice lacking *p57^{KIP2}* indicates a role in Beckwith-Wiedemann syndrome. *Nature* 387:151–158