# Extensive investigation of the *IGF2/H19* imprinting control region reveals novel OCT4/SOX2 binding site defects associated with specific methylation patterns in Beckwith-Wiedemann syndrome

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## Abstract

Isolated gain of methylation (GOM) at the *IGF2/H19* imprinting control region 1 (ICR1) accounts for about 10% of patients with BWS. A subset of these patients have genetic defects within ICR1, but the frequency of these defects has not yet been established in a large cohort of BWS patients with isolated ICR1 GOM. Here, we carried out a genetic analysis in a large cohort of 57 BWS patients with isolated ICR1 GOM and analyzed the methylation status of the entire domain. We found a new point mutation in two unrelated families and a 21 bp deletion in another unrelated child, both of which were maternally inherited and affected the OCT4/SOX2 binding site in the A2 repeat of ICR1. Based on data from this and previous studies, we estimate that *cis* genetic defects account for about 20% of BWS patients with isolated ICR1 GOM. Methylation analysis at eight loci of the *IGF2/H19* domain revealed that sites surrounding OCT4/SOX2 binding site mutations were fully methylated and methylation indexes declined as a function of distance from these sites. This was not the case in BWS patients without genetic defects identified. Thus, GOM does not spread uniformly across the *IGF2/H19* domain, suggesting that OCT4/SOX2 protects against methylation at local sites. These findings add new insights to the mechanism of the regulation of the ICR1 domain. Our

data show that mutations and deletions within ICR1 are relatively common. Systematic identification is therefore necessary to establish appropriate genetic counseling for BWS patients with isolated ICR1 GOM.

## INTRODUCTION

The human chromosome region 11p15.5 encompasses two independent imprinted regions that play a crucial role in the control of fetal and postnatal growth: the telomeric domain *IGF2/H19* is regulated by the Imprinting Control Region 1 (ICR1) that is methylated on the paternal allele during spermatogenesis (germline methylated region), and the centromeric domain *CDK1NC/KCNQ1* is regulated by the ICR2 (germline methylated region), which is methylated on the maternal allele (1). Abnormalities  at the 11p15.5 region lead to two clinical disorders with opposite developmental imbalances: Beckwith–Wiedemann Syndrome (BWS: OMIM 130650), which is an overgrowth disorder associated with a high risk of pediatric tumors, and Russell–Silver Syndrome (RSS: OMIM 180860), which is characterized by severe fetal and postnatal growth retardation associated with body asymmetry and nutritional problems (2,3).

The *IGF2/H19* domain plays a dual role in development and tumorigenesis. It is regulated by ICR1 which is located 2 kb upstream of the *H19* gene. Shared endodermal and mesodermal enhancers downstream from *H19* (4) modulate the transcription of *IGF2* and *H19* genes in an allele–specific manner. *IGF2*, an important growth factors during embryogenesis, is transcribed from the methylated paternal allele, whereas the *H19* gene, which codes for an untranslated RNA, is transcribed from the unmethylated maternal allele (1).

The human ICR1 is organized in two repeated blocks containing A and B repeat elements. It contains seven CpG–rich repeats able to bind the CCCTC–binding factor (CTCF), a highly conserved zinc–finger DNA–binding protein with multiple roles in gene regulation including chromatin insulator function (5,6). A total of six CTCF–binding sites (CBS) are present within B–repeat elements and a seventh binding site is positioned between the repeat blocks and the *H19* transcription start site (7). In addition to these CBS, the *IGF2/H19* domain contains several other differentially methylated regions (DMR): two within the *IGF2* gene (DMR0 and DMR2) and an additional DMR located at the *H19* promoter (*H19*DMR); all of which are secondary DMRs (somatic DMRs) methylated on the paternal allele (8). Several studies have illustrated the complex regulation of the *IGF2/H19* domain both in humans and in animal models (9). Chromatin conformation studies show that the domain undergoes complex allele–specific long–range chromatin interactions that prevent either transcription of *H19* from the paternal allele or transcription of *IGF2* from the maternal allele (10-13). Recent findings from studies with human samples confirm that the loss of imprinting (LOI) at the *IGF2/H19* domain disturbs these long–range chromatin interactions (14-17).

Genetic and/or epigenetic abnormalities at chromosome 11p15.5 account for most cases of BWS. These abnormalities occur through copy–number variations, paternal isodisomy of chromosome 11 (pIPD11) or disruption of regulatory sequences leading to imprinting defects such as gain (ICR1) or loss (ICR2) of DNA methylation. However, genetic defects account only for 25–30% of cases and the cause of most cases of 11p15.5 LOI is unknown. All these anomalies alter the expression of either the maternally or the paternally transcribed genes (18).

Gain of methylation (GOM) occurs within the *IGF2/H19* domain at ICR1 in

about 10% of patients with BWS [19]; this epigenetic anomaly induces the bi-allelic expression of *IGF2* and transcriptional silencing of *H19*. The identification of several duplications, microdeletions or point mutations affecting binding sites of *trans*-acting factors in patients with BWS led to the discovery of some of the mechanisms that regulate genomic imprinting at the *IGF/H19* domain. Indeed, several inherited or *de novo* microdeletions that remove a subset of CBS in the maternal allele have been reported in patients with BWS [7,20-25]. These deletions prevent CTCF binding at ICR1, resulting in alteration of chromatin looping at the maternal allele, thus leading to GOM of the remaining CBS and bi-allelic expression of *IGF2*.

Recent data demonstrate that the function of CTCF is modulated by neighboring DNA-binding factors such as Cohesin and by the pluripotency factors OCT4 and SOX2 [26]. Three point mutations and one deletion within the OCT4/SOX2 binding site in the maternal ICR1 A2 repeat, and a deletion of the SOX2 binding site in the maternal ICR1 B3 repeat have been described in BWS patients with isolated ICR1 GOM [7,27-29]. This suggests that these factors play a role in maintaining or establishing the unmethylated status of the maternal allele. This assumption is strongly supported by *in vitro* experiments and by transgenic murine models that show that the OCT4/SOX2 binding sites within ICR1 are essential to fully protect the maternal ICR1 from DNA methylation during the establishment or maintenance phases [30,31].

The human *IGF2/H19* domain contains three binding sites for the pluripotency factors OCT4 and SOX2 (OCT4/SOX2-motifs: OSM 1,2,3), and all previously identified mutations are located in the OSM2 within the A2 site of ICR1 [7,27-29]. In this study, we report new BWS patients with isolated ICR1 GOM carrying *cis* genetic alterations that affect the OCT4/SOX2 motif OSM2. We also report the frequency of genetic alterations of the ICR1 in a large cohort of BWS patients with isolated ICR1 GOM. We also studied the methylation pattern of the entire *IGF2/H19* domain in great detail to document how GOM spreads across the domain.

## RESULTS

### Imprinting status at the 11p15 region in the BWS cohort

We carried out genetic and methylation studies of the *IGF2/H19* telomeric domain in a large cohort of BWS patients with isolated ICR1 GOM to determine the genetic and epigenetic mechanisms underlying the disease in these patients.

We studied 57 (52 unrelated cases) BWS patients displaying GOM at ICR1 CBS2 [median methylation index (MI) 75%, range 56-100%; normal range 46-56%]. These patients had a normal methylation pattern at ICR2 (median MI 50%, range 46-54%; normal range 41-54%). MI was determined by ASMM RTQ-PCR [32] in DNA extracted from leukocytes and fetal tissues. Twenty-nine of the 57 patients have been previously reported [7,25]. The ICR1 of these patients has been both sequenced and examined for duplication/deletion (dup/del). Twenty-eight of the 57 patients are reported here for the first time.

### Genetic defects affecting the ICR1 and the OCT4/SOX2 motifs

We used a hierarchical approach to search for genetic defects. We first searched for large deletions or duplications affecting the 11p15 region by SNP Oligo Pool Arrays ($n = 29$) [25], SNP microarray ($n = 13$) [33] and/or multiplex ligation dependent probe amplification (MLPA) ($n = 20$). Subsequently, for all the patients showing no duplication or deletion, we sequenced the entire ICR1 (GRCh37/hg19 chr11: 2020201-2024487)

including OSM2 (A2 repeat), OSM3 (A1 repeat) and the SOX2 binding site (B3 repeat). We also sequenced OSM1 which lies outside of ICR1.

Of the 29 patients previously published, 1 patient had a duplication of the telomeric domain, 6 patients had ICR1 partial deletions and a familial case with an OSM2 mutation (7,25). We did not identify any duplications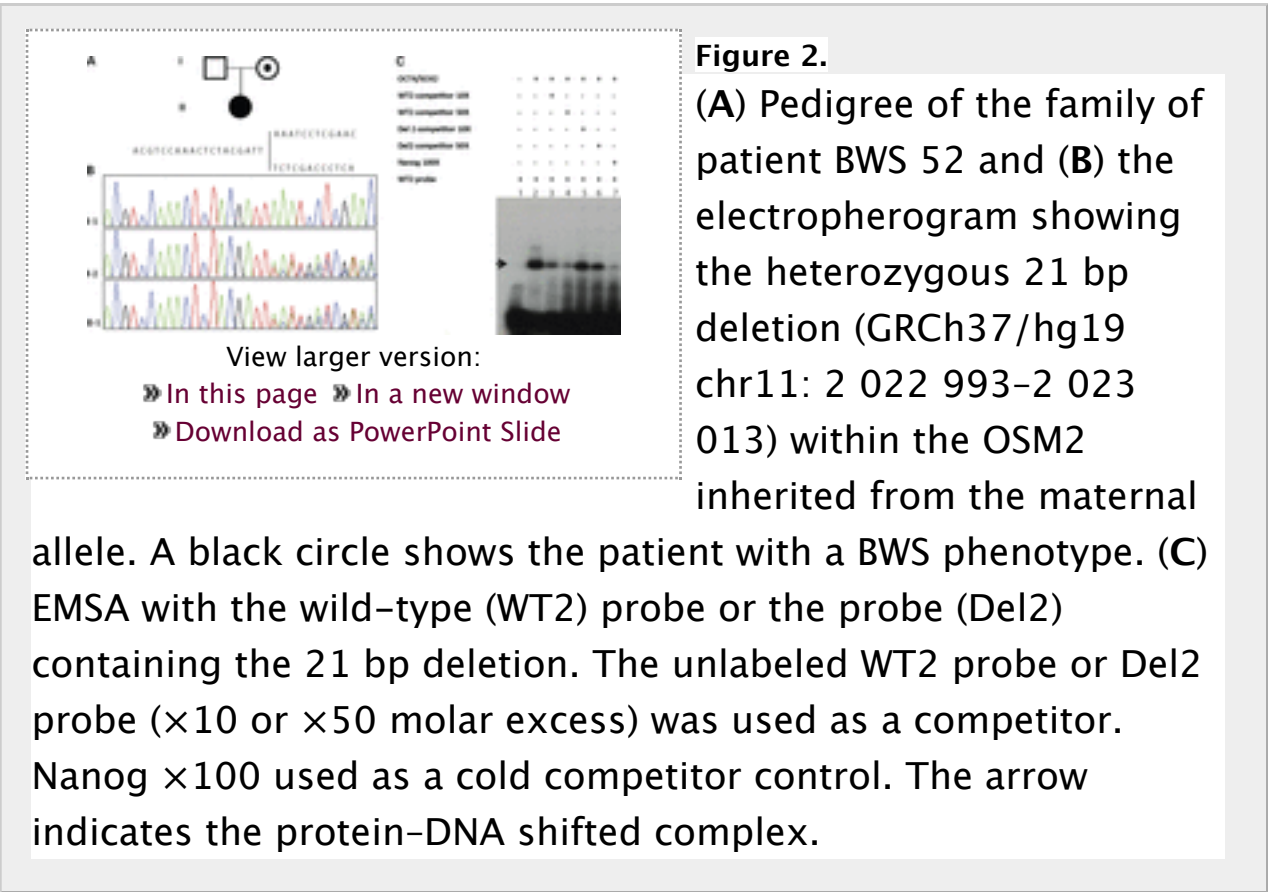 or large deletions in the 28 newly described patients. Interestingly, we identified two new mutation/deletion affecting OSM2 in three independent cases. Two of these cases harbored the same mutation, but patients were born to unrelated and non-consanguineous families. Haplotype analysis and chromosome 11 microsatellite-based linkage studies showed that the two families are not related (data not shown). Several patients with BWS were born to these two families within the same generation (Fig. 1A and B). In one of these families, DNA was only available for patient BWS 54 (family 1 III-3), his unaffected brother (family 1 III-1) and their parents (Fig. 1A). In the other family, DNA was available for patients BWS 3 and BWS 4 (family 2 III-1 and III-2, respectively), their parents, and for patient BWS 10 (family 2 III-3) (Fig. 1B).



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**Figure 1.**
Familial BWS cases. (**A** and **B**) Pedigrees of the BWS family 1 (A) and BWS family 2 (B). Black circles/squares show patients with BWS phenotype. (**C** and **D**) Electropherogram showing the heterozygous mutation (G>A) within the third octamer of OSM2 located within repeat A2 (GRCh37/hg19 chr11:2023045) inherited from the maternal allele for patients BWS 54 (C) and BWS 3, 4, or 10 (D). (E) EMSA with the wild-type (WT1) probe or the mutant (Mut1) probe containing the 2023045 G>A mutation. The unlabeled WT1 probe or Mut1 probe (×10, ×50 or ×200 molar excess) was used as a competitor. Nanog ×100 was used as a cold competitor control. The arrow indicates the protein–DNA shifted complex.

DNA sequencing of the entire ICR1 region showed that patient BWS 54 (family1 III-3) and patients BWS 3, 4, and 10 (family2 III-1, -2, and -3, respectively) carried the same maternally inherited single nucleotide variant (NC_000011.9:g.2023045G>A ss947850191). This variant was located in the third octamer of OSM2 within repeat A2 (Fig. 1C and D). The presence of BWS in several first cousins from the third generation of the pedigree suggests that the mutation was inherited from grandparents, and did not arise *de novo* in the second generation. Grandparental DNA was not available for any of the families to determine the origin of the variant, but both mothers carrying the mutation had normal MIs at ICR1, suggesting that the variant was inherited from the maternal grandfather (Family1 I-1 and Family2 I-1). This variation was not found in the unaffected child of the first family (Family 1 III-1), or in a control population ($n = 100$ chromosomes) or in public databases and is therefore probably a deleterious mutation that affects the binding of OCT4/SOX2 to their site. Another patient (BWS 52) carried a 21 bp deletion (NC_000011.9:g 2022993_2023013 ss947850193) within the OSM2 that resulted in the fusion of the first and the second octamers of OSM2. This deletion was also maternally inherited (Fig. 2A and B). Grandparental DNA

was not available to determine the origin of the deletion. However, given that the mother carrying the deletion is healthy and no other BWS cases were recorded in her extended family suggest that (i) the deletion occurred *de novo* in the maternal grandfather gamete; or (ii) the deletion was inherited from the maternal grandfather.



**Figure 2.**
(**A**) Pedigree of the family of patient BWS 52 and (**B**) the electropherogram showing the heterozygous 21 bp deletion (GRCh37/hg19 chr11: 2 022 993–2 023 013) within the OSM2 inherited from the maternal allele. A black circle shows the patient with a BWS phenotype. (**C**) EMSA with the wild-type (WT2) probe or the probe (Del2) containing the 21 bp deletion. The unlabeled WT2 probe or Del2 probe (×10 or ×50 molar excess) was used as a competitor. Nanog ×100 used as a cold competitor control. The arrow indicates the protein-DNA shifted complex.

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Sequencing of the ICR1 domain also showed other previously unreported variants in patients with BWS (Table 1).

**Table 1.**
Sequence variations of the ICR1 region in BWS patients

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*Clinical features*

BWS familial case 1 (Fig. 1A, Table 2): all individuals, except for one (III–1), from the third generation of this family showed a BWS phenotype, particularly neonatal macrosomia. Patient BWS 54 (III–3) is the third child of non-consanguineous parents. He was born with macrosomia (birth weight 4880 g +3.5 SDS), macroglossia, organomegaly and a nephromegaly that later developed into a Wilms' Tumor. His eldest brother (III–1) did not display any BWS phenotype, whereas his other brother (III–2) died a few days after birth due to heart failure. Two maternal first cousins of these patients also had symptoms resembling those of patient BWS 54 (macrosomia, macroglossia and kidney tumors). Medical termination of pregnancy was carried out for individual III–5 due a BWS phenotype and Klinefelter syndrome (47, XXY).

**Table 2.**
Clinical features of BWS patients with ICR1 genetic defects

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BWS familial case 2 (Fig. 1B, Table 2): two sisters (BWS 3 and BWS 4) and one maternal first cousin (BWS 10) had clinical features typical of BWS. Patient III–1 (BWS3) was born after 40 weeks of gestation to non-consanguineous parents. The clinical presentation at birth included macrosomia (birth weight 4780 g, +2.8 SDS), macroglossia, ear abnormalities, diastasis recti and umbilical hernia. Her sister (III–2) displayed a BWS phenotype *in utero*, and was born after 37 weeks of gestation with macrosomia (birth weight 4230 g, +3.6 SDS), macroglossia and organomegaly. Patient BWS 10 (III–3) was born after 30 weeks of

gestation to non-consanguineous parents following an emergency Caesarean section due to a decrease in the movements of the fetus. This patient died a few hours after birth due to heart failure. Post-mortem examination showed several clinical signs of BWS, including severe macroglossia and a nephromegaly.

BWS case 3 (Fig. 2A, Table 2): patient BWS 52 was born to non-consanguineous parents and showed typical clinical signs of BWS: macrosomia (birth weight 4590 g), macroglossia and umbilical hernia. No other BWS cases were recorded in the extended family of either the mother or the father.

Clinical features of other patients with ICR1 genetic defects in this cohort that have been previously published are listed in Table 2.

**Functional study of the OCT4/SOX2 binding site mutations**

We used an electrophoretic mobility shift assay (EMSA) to determine whether the single nucleotide variant (NC_000011.9:g.2023045G>A ss947850191) or the 21 bp deletion (NC_000011.9:g 2022993_2023013 ss947850193) disrupted the binding of the OCT4/SOX2 complex to its binding sites.

Figure 1E shows EMSA results obtained with protein extracts from cells overexpressing human OCT4/SOX2 and a wild-type $^{32}$P-labelled probe. OCT4/SOX2 interacted with the wild-type $^{32}$P-labelled probe and induced a band shift seen in lane 2. This interaction was efficiently competed by an excess of unlabeled wild-type competitor probe (lanes 3-5). However, the interaction of wild-type $^{32}$P-labelled probe with OCT4/SOX2 was weakly competed by an excess of unlabeled mutant competitor probe carrying the G>A variant (lanes 6-9). Similar results were obtained with an excess of unlabeled competitor probe harboring the 21 bp deletion (Fig. 2C).

These results, in addition to maternal inheritance of the G>A mutation, as well as the absence of this variation in the unaffected brother of family 1, and the 21 bp deletion, strongly suggest that these variants alter the binding of OCT4/SOX2 *in vivo* and therefore are associated with the GOM at ICR1.

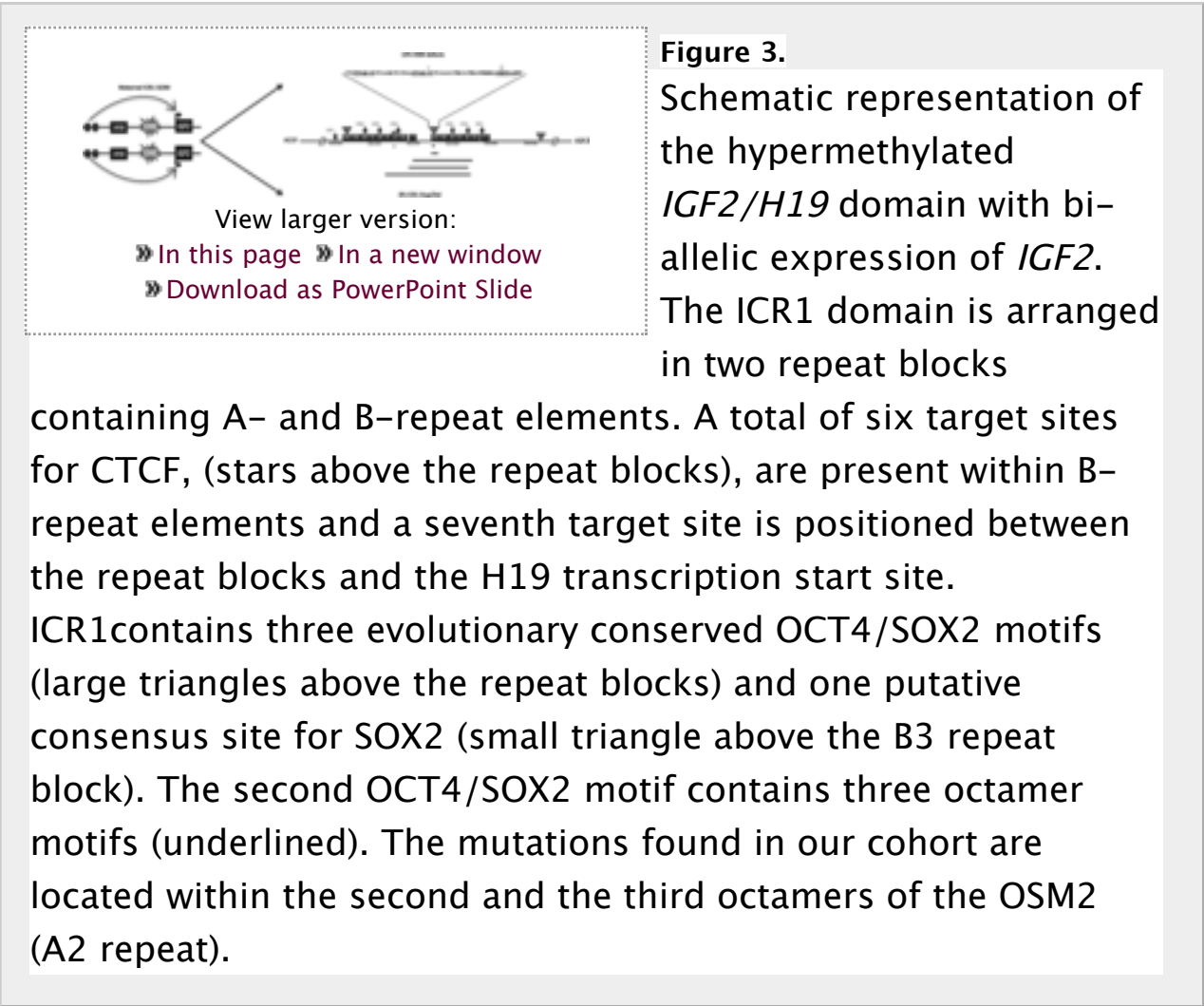**Frequencies of genetic alterations in BWS patients with isolated ICR1 GOM**

This work is a continuation of a previous study involving BWS patients included in this cohort (7,25). The 29 patients previously investigated included a family with two affected members [BWS 15 and 16 in (7)] who were the first reported cases of BWS patients with mutation in OCT4/SOX2 binding sites in ICR1. Six other previously reported patients had ICR1 microdeletions [BWS 5, 12, 13 in (7)], including two familial cases [H63P, this patient's mother, and H50P in (25) and her mother, BWS 51] and one duplication of the telomeric domain [H47P in (25)]. In this study, we report two new familial cases of a novel point mutation in the OCT4/SOX2 motif (OSM2), and a small 21 bp deletion within the same site.

We calculated the frequencies of *cis* genetic alterations within ICR1 that are both associated with BWS and transmitted to the next generation. We found six cases of OCT4/SOX2 binding site variants (five within the OSM2 in the A2 repeat and one small deletion of the SOX2 motif in the B3 repeat) corresponding to ~12% (6/52, excluding the related patients) of the cohort. We also found three large microdeletions, all of which were included the A2 repeat containing the OSM2, and one duplication of the telomeric domain, representing ~8% (4/52) of the cohort.

Altogether, these data show that around 20% (10/52) of BWS patients with isolated ICR1 GOM carry genetic alterations that affect the *IGF2/H19* ICR1 domain. All of these alterations, except the whole *IGF2/H19* ICR1 domain
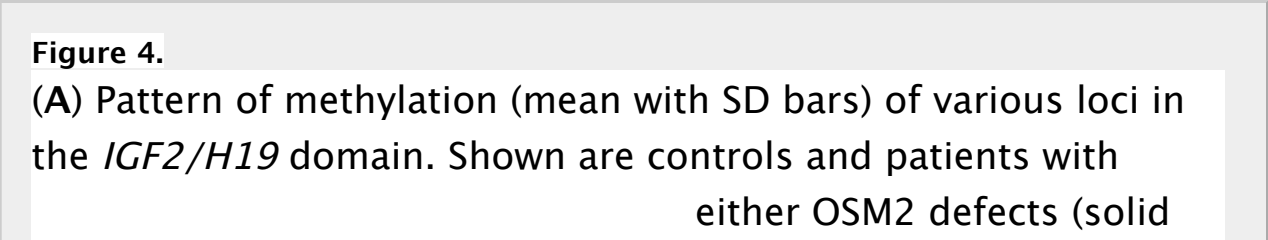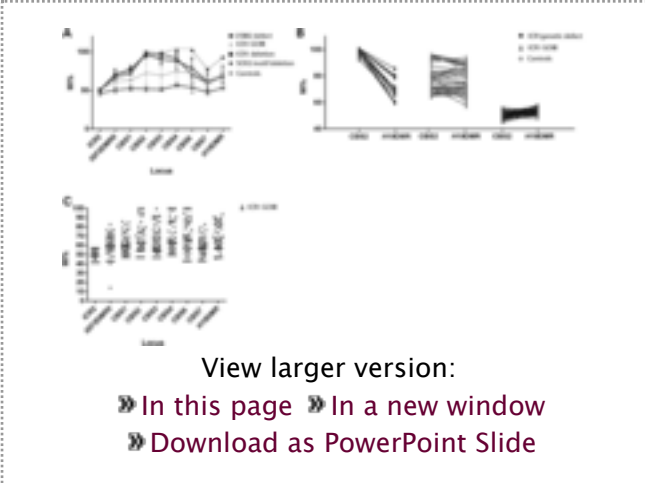
duplication, affect either OCT4/SOX2 binding sites within the A2 repeat or the SOX2 motif in the B3 repeat (Fig. 3).



**Figure 3.**
Schematic representation of the hypermethylated *IGF2/H19* domain with bi-allelic expression of *IGF2*. The ICR1 domain is arranged in two repeat blocks containing A- and B-repeat elements. A total of six target sites for CTCF, (stars above the repeat blocks), are present within B-repeat elements and a seventh target site is positioned between the repeat blocks and the H19 transcription start site. ICR1contains three evolutionary conserved OCT4/SOX2 motifs (large triangles above the repeat blocks) and one putative consensus site for SOX2 (small triangle above the B3 repeat block). The second OCT4/SOX2 motif contains three octamer motifs (underlined). The mutations found in our cohort are located within the second and the third octamers of the OSM2 (A2 repeat).

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**Impact of OCT4/SOX2 mutations and ICR1 microdeletions on the *IGF2/H19* ICR1 methylation profile**

We studied the effect of the OCT4/SOX2 binding site alterations and ICR1 microdeletions on the methylation profile at the *IGF2/H19* ICR1 domain. We used Allele-Specific Methylated Multiplex Real-time Quantitative PCR to examine the methylation status at *IGF2*DMR0, CBS 1, 2, 3, 4, 6, 7 and at *H19*DMR (32). ICR1 CBS5 was not analyzed due to a SNP (rs113013264) within the hybridization site of the TaqMan probes targeting CBS5. We found almost a complete GOM at CBSs surrounding the OSM2 mutations ($n = 8$), especially at CBS2 and CBS3. MI declined as a function of distance from these binding sites, attaining its lowest levels near *IGF2*DMR0 and *H19*DMR; this pattern of methylation looks like a downward crescent, or a 'moustache' (Fig. 4A). We found a similar methylation profile in five patients carrying ICR1 microdeletions. Patients carrying a 2.2 or 1.8 kb microdeletion within the ICR1 displayed partial GOM at the remaining CBSs and DMRs (Supplementary Material, Table S1). Indeed, patients BWS 35, 50 and 51, who carry a 1.8 kb deletion that removes only CBS2 and CBS3, showed almost complete GOM at CBS4. Interestingly, this hypermethylation profile was shifted to the right in patient BWS 16, who carries an 8 bp deletion that removes the SOX2 binding site within the B3 repeat. Indeed, this patient showed complete GOM at CBS4 and CBS6, and a MI = 91% at the *H19*DMR (Fig. 4A and Supplementary Material, Table S1). In contrast, patients who do not have OSM2 mutations or ICR1 microdeletions ($n = 43$) showed a consistent pattern of methylation across this region, suggesting that the GOM is evenly distributed across the entire domain (Fig. 4A). For instance, there was a large gradient in MI between CBS2 and *H19*DMR in patients with an ICR1 genetic defect (OSM2 mutations or ICR1 microdeletions) (CBS2 MI = 98 ± 2.8% versus *H19*DMR MI = 70 ± 8.5%). However, this was not the case for other BWS patients with isolated ICR1 GOM (CBS2 MI = 73 ± 14% versus *H19*DMR MI = 71 ± 12%) (Fig. 4B).

**Figure 4.**
(**A**) Pattern of methylation (mean with SD bars) of various loci in the *IGF2/H19* domain. Shown are controls and patients with either OSM2 defects (solid

black line), SOX2 binding site deletion (dotted black line), ICR1 deletions (dark gray line) and patients without any ICR1 genetic defect 'ICR1 GOM' (light grey line). MI across the *IGF2/H19* domain in patients with OSM2 defects or ICR1 deletions has a 'moustache-like' appearance, whereas MI in patients without ICR1 genetic defects is uniform across this region. (**B**) Comparison of ICR1 CBS2 and *H19*DMR MIs in controls and BWS patients with or without ICR1 genetic defects. There is a large difference in MI at ICR1 CBS2 and *H19*DMR in patients carrying a genetic defect within the ICR1, whereas MI at these two positions is similar in BWS patients showing isolated ICR1 GOM without any ICR1 genetic defect. (**C**) Distribution of the MI at different loci within the *IGF2/H19* domain. Shown are all BWS patients with ICR1 CBS2 GOM who do not carry ICR1 genetic defects. Horizontal lines show the normal range of MI for each position (mean ± 2 SD).

*IGF2/H19* **ICR1 domain methylation pattern in BWS patients with 'sporadic' ICR1 GOM**

We further sought to study how the GOM spreads across the *IGF2/H19* ICR1 domain in patients without any identified genetic defects within the ICR1. We measured the MI at the *IGF2*DMR0, the ICR1 CBSs (excluding ICR1 CBS5) and the *H19*DMR in patients with BWS showing ICR1 CBS2 GOM. We found that all loci were not affected in the same way (Fig. 4C). The MI at ICR1 CBS2, CBS3 and *H19*DMR was similar. In contrast, GOM at *IGF2*DMR0, ICR1 CBS1, CBS4, CBS6 and CBS7 showed a large degree of inter-individual variability and was less severe than hypermethylation that occurred at ICR1 CBS2. Indeed, MI values were often in the normal range at these loci: the proportion of patients with normal values was 19% at I*GF2*DMR0, 37% at CBS1 and CBS6 and 28% at CBS4 and CBS7. Interestingly, patients BWS 20 and BWS 24 had partial demethylation at *IGF2*DMR0 (MI was 15% for BWS 20 and 41% for BWS 24; normal range 43–57%). The possibility that a SNP had interfered with the hybridization of TaqMan probes was ruled out by direct sequencing of the amplification products. These results suggest that all ICR1 CBSs, *IGF2*DMR0 and *H19*DMR do not acquire the same level of methylation during the *de novo* phase of methylation, due to a defect of maternal allele methylation. The most affected loci are CBS2, CBS3 and *H19*DMR (Fig. 4C).

## DISCUSSION

The 11p15.5 imprinted region is an exemplary model to investigate the regulation of genomic imprinting. Although it has been extensively studied, the fine-tuning of the regulation of this domain is still poorly understood. Isolated GOM at the *IGF2/H19* ICR1 imprinted domain accounts for about 10% of cases of BWS (19). GOM was considered as a sporadic event that was not transmissible to subsequent generations, until the first published cases of ICR1 deletion in patients with BWS (22). In this study, we investigated ICR1 genetic defects in the largest cohort of BWS patients with isolated ICR1 GOM reported so far. We found a new maternally transmitted point mutation (NC_000011.9:g.2023045G>A ss947850191) in patients from two unrelated families. We also found a

maternally transmitted small 21 bp deletion (NC_000011.9:g 2022993_2023013 ss947850193) in another patient. Both the point mutation and the small deletion affected the OCT4/SOX2 motif within the A2 repeat of ICR1. Functional tests revealed that these defects impaired the binding of OCT4/SOX2 at their sites, which probably resulted in the GOM on the maternal allele.

Indeed, recent studies involving murine models highlight the role of the highly conserved OCT4/SOX2 motif within the mouse ICR1. The OCT4/SOX2 complex has a protective function and cooperates with CTCF to maintain the unmethylated status of the maternal ICR1 (30,31,34,35). The human *IGF2/H19* domain contains three OCT4/SOX2 motifs; however, all genetic defects described so far, including three previously described point mutations (7,27–29) and the mutation and the small deletion that we describe here, are located in the OCT4/SOX2 motif within the ICR1 A2 repeat. These findings suggest that OSM2 is more likely than OSM1 and 3 to play a role in protecting the maternal ICR1 from methylation. Alternatively, OSM2 may be highly prone to mutational events.

We examined the prevalence of the genetic defects that we previously reported (7,25) and those that we report here in a cohort of BWS patients with isolated ICR1 GOM. A genetic defect of the ICR1 was a frequent finding: around 20% of patients were affected; 12% had OSM defects and 8% had ICR1 deletions. We did not find any example [in our cohort or in published cases (20,36)] of a deletion that removed one or several CBS without affecting the OCT4/SOX2 site in the A2 repeat or the SOX2 motif in the B3 repeat. Therefore, it is not known if a deletion that removes a subset of CBS without affecting the OCT4/SOX2 site would influence DNA methylation. We report six previously unidentified variants within the consensus motif of CTCF in CBS2, 4, 5 or 6 in our cohort of patients with BWS. However, we do not know if a particular variant affecting one of the seven CBS can disrupt the establishment of the CTCF complex at ICR1 and lead to GOM. A recent study with F9 cells showed that the protective activity of CTCF was disrupted only if both the CTCF and OCT4/SOX2 sites were affected (30). However, other studies in mice showed that the maternal transmission of an ICR with four mutant CTCF sites results in substantial abnormal methylation across the ICR after implantation (37,38), while another study showed that a deletion removing a sequence of ~0.9 kb between CBS2 and CBS3 at the murine ICR1 (including the Oct–binding site) induced biallelic *H19* expression but not GOM at the maternal ICR1 allele (39). Furthermore, the fact that the deletion including the Oct–binding site did not induce ICR1 hypermethylation upon maternal transmission questioned the role of OCT4/SOX2 in the regulation of ICR1 on the maternal allele in the mouse. Hence, further studies are necessary to decipher the proper OCT4/SOX2 role in the protection of the maternal ICR1 allele against gaining methylation during the postimplantation period.

We further studied the ICR1 GOM defect across the entire *IGF2/H19* domain. We found that OCT4/SOX2 mutations resulted in a complete GOM at ICR1 CBS2 and CBS3, whereas other distant CBSs, *IGF2*DMR0 and *H19*DMR only showed a partial GOM. These methylation patterns were similar in leukocytes from seven patients and in one tissue (kidney). This result is consistent with experiments involving a mouse model that showed that most maternally inherited mutated alleles resulted in a complete GOM at sites near the OCT4/SOX2 motif, whereas distant sites were less affected (30). Therefore, it appears that OSM defects result in strong hypermethylation of the maternal allele specifically at these sites, and that hypermethylation declines as a function of distance from the

OSM. Thus, the effect of these mutations is a targeted event rather than a stochastic one as previously suggested (27,28). In contrast to these studies, we carried out almost exhaustive analysis of the methylation pattern at the *IGF2/H19* domain and found partial MIs at *IGF2*DMR0, *H19*DMR and ICR1 CBS6 consistent with these previous findings (27,28). However, we found that ICR1 CBS2 and CBS3 were fully methylated in a subset of patients carrying an OSM defect. Thus, our results demonstrate that the effect of OSM2 mutations on GOM is targeted, ruling out the hypothesis of a stochastic GOM. The methylation profile at ICR1 is similar between patients carrying a 2.2 kb (affecting CBS2, CBS3 and CBS4) or a 1.8 kb (affecting only CBS2 and CBS3) deletion within ICR1 and patients carrying an OSM2 mutation. This suggests that the remaining ICR1 CBSs in the maternal allele gained methylation probably due to the loss of OCT4/SOX2 binding. Interestingly, in patient BWS 16, the microdeletion of 8 bp affecting the SOX2 binding site located within the B3 repeat, also resulted in a local effect. In this patient, the GOM pattern observed, compared with those observed in patients with OSM2 mutations or microdeletions, was shifted towards CBS4 and CBS6. These findings suggest that OCT4 and SOX2 may be involved in controlling the methylation of the *IGF2/H19* domain. These mutations probably lead to GOM by causing chromatin conformational changes. Indeed, interaction of OCT4 with Cohesin and CTCF at CBS5 of the homeobox gene A locus promotes gene activation by looping the intervening DNA sequences between these elements (40). The organization of the homeobox gene A locus and ICR1 is similar. Based on this observation and on our methylation results, we hypothesize that OCT4/SOX2, together with CTCF and Cohesin, may contribute to the formation of a chromatin loop involving a subset of CBS. If OSM2 is altered, CBS2 and CBS3 are likely to become exposed and hence fully methylated, whereas other CBSs, probably inside the loop, are less exposed and are thereby only partially methylated.

It is important to point out that BWS patients with OSM2 genetic defects show a clinical spectrum. Even those carrying the same point mutation (BWS 3, 4, 10 and 54) and those who show similar MIs display some BWS phenotypic differences, involving abdominal wall defects, hypoglycemia, ear abnormalities and Wilms' tumor. Nonetheless, almost all of these patients share some of the major features of BWS like macrosomia, macroglossia and organomegaly.

In the absence of an ICR1 genetic defect, all loci are not methylated in the same way, and some of them are methylated at normal levels. CBS2, CBS3 and *H19*DMR appear to be the most affected in all BWS patients with isolated ICR1 GOM. These results suggest that (i) different mechanisms may be involved in the occurrence of GOM; and (ii) other potential *trans*-acting factors may regulate the establishment of DNA methylation during the phase of *de novo* methylation after implantation.

In conclusion, we studied the genetic and epigenetic patterns (methylation profiles at most *IGF2/H19* DMRs and ICR1 CBSs) in the largest reported cohort of BWS patients with isolated ICR1 GOM. Our objective was to find new genetic defects and to characterize the pattern of GOM occurring in BWS patients, and its dependence on OCT4/SOX2 binding sites mutations.

We show that OCT4/SOX2 mutations and deletions within ICR1 are common in patients with BWS and account for ~20% of BWS patients with isolated ICR1 GOM. Six out of 10 of these variants were point mutations or small deletions affecting the OCT4/SOX2 motifs. OSM2 mutations are associated with diminished binding of OCT4/SOX2 as determined by EMSAs *in vitro* and also associated with complete GOM of ICR1 CBS2 and

CBS3 *in vivo*. Direct sequencing of the 11p15 ICR1 region should be incorporated into routinely used diagnostic tools to establish accurate methods of genetic counseling. This is especially important for patients who show large variation in MIs at CBS2 and *H19*DMR.

## MATERIALS AND METHODS

### Population studied

Controls: we used ASMM RTQ–PCR to analyze the methylation status of ICR1 and ICR2 on 11p15 region in 30 healthy individuals.

Ethical considerations: written informed consent for participation was received for all patients ($n = 57$), either from the patients themselves or their parents, in accordance with French national ethics rules for patients recruited in France (Assistance Publique—Hôpitaux de Paris authorization no. 681).

Patients were either followed at Armand Trousseau Children's Hospital or were referred by other clinical centers for molecular analysis of suspected BWS. A geneticist and/or a pediatric endocrinologist examined each patient and a detailed clinical form was completed.

All patients displayed 11p15.5 isolated ICR1 GOM. Blood samples were collected during clinical visits.

### Nucleic acid extraction

DNA was extracted from blood leukocytes by an in-house protocol as previously described (41), and from fetal tissues conserved in liquid nitrogen (kidney, lung and muscle) that were obtained from a post mortem examination. Fetal tissues were ground in the presence of dry ice until a powder was obtained. DNA was then extracted from all the tissues after cell lysis by a salting out procedure, as previously described (42).

### Bisulfite treatment of DNA

Sodium bisulfite DNA treatment converts all the unmethylated cytosine residues to uracil residues. The methylated cytosine residues are unaffected. This process thus generates C/T polymorphisms, which can be used to distinguish between the methylated and the unmethylated allele. Genomic DNA (1 μg) was treated with sodium bisulfite, with the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA), according to the manufacturer's instructions. Genomic DNA was eluted with 50 μl RNase-free $H_2O$ and conserved at −20°C.

### TaqMan allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR) and methylation analysis

The methylation status of the ICR1 CTCF binding sites (CBS1, 2, 3, 4, 6 and 7), the *H19* promoter (*H19*DMR) (GenBank AF125183) and *IGF2*DMR0 (GenBank AC132217) at 11p15.5 were assessed by ASMM RTQ–PCR, as previously described (32). ICR1 CBS5 was excluded from the analysis because of a SNP (rs113013264), located within the hybridization sites of the ASMM RTQ–PCR TaqMan probes, which gave variable results even in the control population (data not shown). The MI at each locus was assigned by calculating the ratio between the methylated and unmethylated alleles as follows: (amount of methylated allele/sum of both methylated and unmethylated alleles)×100. The ASMM RTQ–PCR primers and probes sequences are available on request.

### Multiplex ligation dependent probe amplification

MLPA analysis was performed using the methylation specific MLPA kit ME030 version C3 from MRC-Holland (Amsterdam, The Netherlands) following the manufacturer's instructions. The amount of genomic DNA

used for the analysis was 200 ng.

**SNP microarray analysis**

Uniparental disomy and large copy number variations were ruled out by experiments with Illumina CytoSNP–12 arrays (Illumina, San Diego, CA, USA). Briefly, samples were processed with the Infinium assay and results were analyzed with the Illumina GenomeStudio software. References were built with the clustering algorithm Illumina Gentrain 2.0 with SNP profiles from 82 control samples.

**OCT4/SOX2 motifs and ICR1 sequencing**

OSM1, ICR1 (including CBS 1 to 6, OSM 2 and 3) and CBS7 were sequenced by standard methods of Sanger sequencing, using the ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer. The sequencing products were then analyzed with SeqScape v2.6 (Lifetechnologies, Courtaboeuf, France). The PCR and sequencing oligonucleotides are listed in Supplementary Material, Table S2.

**Electrophoretic mobility shift assays (EMSA)**

HEK cells (Invitrogen) were transfected with 1 µg of each pPS–humanOct4–T2A–RFP and pPS–human Sox2–T2A–RFP constructs (System Bioscience, CA, USA) by Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Forty–eight hours after transfection, cell lysates were prepared from the cells producing human OCT4 and SOX2 proteins (OCT4/SOX2), with complete M–lysis buffer (Roche Applied Science, France). The EMSA oligonucleotides are listed in Supplementary Material, Table S2. Labeled double–stranded probes (30 fmoles) were incubated for 30 min at room temperature with 5 µg of protein extracts from cell lysates containing OCT4/SOX2 proteins in a buffer containing 12 mM HEPES, 60 mM KCl, 2 mM EDTA, 1 mM DTT, 3 mg of BSA and 1 mg poly[d(I–C)] in a volume of 20 µl. The reaction mixtures were separated on a 4% polyacrylamide gel, which was dried and exposed overnight to a film at −80°C.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

## FUNDING

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## Notes

## REFERENCES

1. Demars J., Le Bouc Y., El-Osta A., Gicquel C. Epigenetic and genetic mechanisms of abnormal 11p15 genomic imprinting in Silver–Russell and Beckwith–Wiedemann syndromes. Curr. Med. Chem. 2011;18:1740–1750.
   » CrossRef » Medline » Google Scholar

2. Gicquel C., Rossignol S., Cabrol S., Houang M., Steunou V., Barbu V., Danton F., Thibaud N., Le Merrer M., Burglen L., et al. Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver–Russell syndrome. Nat. Genet. 2005;37:1003–1007.
   » CrossRef » Medline » Web of Science » Google Scholar

3. Netchine I., Rossignol S., Dufourg M.N., Azzi S., Rousseau A., Perin L., Houang M., Steunou V., Esteva B., Thibaud N., et al. 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell–Silver syndrome: clinical scoring system and epigenetic–phenotypic correlations. J. Clin. Endocrinol. Metab. 2007;92:3148–3154.
   » CrossRef » Medline » Web of Science » Google Scholar

4. Leighton P.A., Saam J.R., Ingram R.S., Stewart C.L., Tilghman S.M. An enhancer deletion affects both H19 and Igf2 expression. Genes Dev. 1995;9:2079–2089.
   » Abstract/FREE Full Text

5. Frevel M.A., Sowerby S.J., Petersen G.B., Reeve A.E. Methylation sequencing analysis refines the region of H19 epimutation in Wilms tumor. J. Biol. Chem. 1999;274:29331–29340.
   » Abstract/FREE Full Text

6. Takai D., Gonzales F.A., Tsai Y.C., Thayer M.J., Jones P.A. Large scale mapping of methylcytosines in CTCF–binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. Hum. Mol. Genet. 2001;10:2619–2626.
   » Abstract/FREE Full Text

7. Demars J., Shmela M.E., Rossignol S., Okabe J., Netchine I., Azzi S., Cabrol S., Le Caignec C., David A., Le Bouc Y., et al. Analysis of the IGF2/H19 imprinting control region uncovers new genetic defects, including mutations of OCT–binding sequences, in patients with 11p15 fetal growth disorders. Hum. Mol. Genet. 2010;19:803–814.
   » Abstract/FREE Full Text

8. Monk D., Sanches R., Arnaud P., Apostolidou S., Hills F.A., Abu-Amero S., Murrell A., Friess H., Reik W., Stanier P., et al. Imprinting of IGF2 P0 transcript and novel alternatively spliced INS-IGF2 isoforms show differences between mouse and human. Hum. Mol. Genet. 2006;15:1259–1269.
   » Abstract/FREE Full Text

9. Shmela M.E., Gicquel C.F. Human diseases versus mouse models: insights into the regulation of genomic imprinting at the human 11p15/mouse distal chromosome 7 region. J. Med. Genet. 2013;50:11–20.
   » Abstract/FREE Full Text

10. Ribeiro de Almeida C., Stadhouders R., Thongjuea S., Soler E., Hendriks R.W. DNA–binding factor CTCF and long–range gene interactions in V(D)J recombination and oncogene activation. Blood 2012;119:6209–6218.
   » Abstract/FREE Full Text

11. Kurukuti S., Tiwari V.K., Tavoosidana G., Pugacheva E., Murrell A., Zhao Z., Lobanenkov V., Reik W., Ohlsson R. CTCF binding at the H19 imprinting control region mediates maternally inherited higher–order chromatin conformation to restrict enhancer access to Igf2. Proc. Natl. Acad. Sci. USA 2006;103:10684–10689.
   » Abstract/FREE Full Text

12. Murrell A., Heeson S., Reik W. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nat. Genet. 2004;36:889–893.
   » CrossRef » Medline » Web of Science » Google Scholar

13. Qiu X., Vu T.H., Lu Q., Ling J.Q., Li T., Hou A., Wang S.K., Chen H.L., Hu J.F., Hoffman A.R. A complex deoxyribonucleic acid looping configuration associated with the silencing of the maternal Igf2 allele. Mol. Endocrinol. 2008;22:1476–1488.
    » CrossRef » Medline » Web of Science » Google Scholar

14. Nativio R., Wendt K.S., Ito Y., Huddleston J.E., Uribe-Lewis S., Woodfine K., Krueger C., Reik W., Peters J.M., Murrell A. Cohesin is required for higher-order chromatin conformation at the imprinted IGF2-H19 locus. PLoS Genet. 2009;5:e1000739.
    » CrossRef » Medline » Google Scholar

15. Zhang H., Niu B., Hu J.F., Ge S., Wang H., Li T., Ling J., Steelman B.N., Qian G., Hoffman A.R. Interruption of intrachromosomal looping by CCCTC binding factor decoy proteins abrogates genomic imprinting of human insulin-like growth factor II. J. Cell Biol. 2011;193:475–487.
    » Abstract/FREE Full Text

16. Vu T.H., Nguyen A.H., Hoffman A.R. Loss of IGF2 imprinting is associated with abrogation of long-range intrachromosomal interactions in human cancer cells. Hum. Mol. Genet. 2010;19:901–919.
    » Abstract/FREE Full Text

17. Nativio R., Sparago A., Ito Y., Weksberg R., Riccio A., Murrell A. Disruption of genomic neighbourhood at the imprinted IGF2-H19 locus in Beckwith-Wiedemann syndrome and Silver-Russell syndrome. Hum. Mol. Genet. 2011;20:1363–1374.
    » Abstract/FREE Full Text

18. Azzi S., Brioude F., Le Bouc Y., Netchine I. Human imprinting anomalies in Fetal and Childhood Growth Disorders: clinical implications and molecular mechanisms. Curr. Pharm. Des. 2013;20:1751–1763.
    » Google Scholar

19. Brioude F., Lacoste A., Netchine I., Vazquez M.P., Auber F., Audry G., Gauthier-Villars M., Brugieres L., Gicquel C., Le Bouc Y., et al. Beckwith-Wiedemann syndrome: growth pattern and tumor risk according to molecular mechanism, and guidelines for tumor surveillance. Horm. Res. Paediatr. 2013;80:457–465.
    » CrossRef » Medline » Google Scholar

20. Beygo J., Citro V., Sparago A., De Crescenzo A., Cerrato F., Heitmann M., Rademacher K., Guala A., Enklaar T., Anichini C., et al. The molecular function and clinical phenotype of partial deletions of the IGF2/H19 imprinting control region depends on the spatial arrangement of the remaining CTCF-binding sites. Hum. Mol. Genet. 2013;22:544–557.
    » Abstract/FREE Full Text

21. Prawitt D., Enklaar T., Gartner-Rupprecht B., Spangenberg C., Oswald M., Lausch E., Schmidtke P., Reutzel D., Fees S., Lucito R., et al. Microdeletion of target sites for insulator protein CTCF in a chromosome 11p15 imprinting center in Beckwith-Wiedemann syndrome and Wilms' tumor. Proc. Natl Acad. Sci. USA 2005;102:4085–4090.
    » Abstract/FREE Full Text

22. Sparago A., Cerrato F., Vernucci M., Ferrero G.B., Silengo M.C., Riccio A. Microdeletions in the human H19 DMR result in loss of IGF2 imprinting and Beckwith-Wiedemann syndrome. Nat. Genet. 2004;36:958–960.
    » CrossRef » Medline » Web of Science » Google Scholar

23. Sparago A., Russo S., Cerrato F., Ferraiuolo S., Castorina P., Selicorni A., Schwienbacher C., Negrini M., Ferrero G.B., Silengo M.C., et al. Mechanisms causing imprinting defects in familial Beckwith-Wiedemann syndrome with Wilms' tumour. Hum. Mol. Genet. 2007;16:254–264.
    » Abstract/FREE Full Text

24. Azzi S., Abi Habib W., Netchine I. Beckwith-Wiedemann and Russell-Silver Syndromes: from new molecular insights to the comprehension of imprinting regulation. Curr. Opin. Endocrinol. Diabetes Obes. 2014;21:30–38.
    » CrossRef » Medline » Google Scholar

25. Demars J., Rossignol S., Netchine I., Lee K.S., Shmela M., Faivre L., Weill J., Odent S., Azzi S., Callier P., et al. New insights into the pathogenesis of Beckwith-Wiedemann and Silver-Russell syndromes: contribution of small copy number variations to 11p15 imprinting defects. Hum. Mutat. 2011;32:1171–1182.
    » CrossRef » Medline » Google Scholar

26. Weth O., Renkawitz R. CTCF function is modulated by neighboring DNA binding factors. Biochem. Cell. Biol. 2011;89:459–468.

» CrossRef » Medline » Google Scholar

27. Poole R.L., Leith D.J., Docherty L.E., Shmela M.E., Gicquel C., Splitt M., Temple I.K., Mackay D.J. Beckwith–Wiedemann syndrome caused by maternally inherited mutation of an OCT-binding motif in the IGF2/H19-imprinting control region, ICR1. Eur. J. Hum. Genet. 2011;20:240–243.
» Medline » Google Scholar

28. Higashimoto K., Jozaki K., Kosho T., Matsubara K., Fuke T., Yamada D., Yatsuki H., Maeda T., Ohtsuka Y., Nishioka K., et al. A novel de novo point mutation of the OCT-binding site in the IGF2/H19-imprinting control region in a Beckwith–Wiedemann syndrome patient. Clin. Genet 2013. DOI:10.1111/cge.12318.

29. Berland S., Appelback M., Bruland O., Beygo J., Buiting K., Mackay D.J., Karen Temple I., Houge G. Evidence for anticipation in Beckwith–Wiedemann syndrome. Eur. J. Hum. Genet. 2013;21:1344–1348.
» CrossRef » Medline » Google Scholar

30. Zimmerman D.L., Boddy C.S., Schoenherr C.S. Oct4/Sox2 binding sites contribute to maintaining hypomethylation of the maternal igf2/h19 imprinting control region. PLoS ONE 2013;8:e81962.
» CrossRef » Medline » Google Scholar

31. Sakaguchi R., Okamura E., Matsuzaki H., Fukamizu A., Tanimoto K. Sox-Oct motifs contribute to maintenance of the unmethylated H19 ICR in YAC transgenic mice. Hum. Mol. Genet. 2013;22:4627–4637.
» Abstract/FREE Full Text

32. Azzi S., Steunou V., Rousseau A., Rossignol S., Thibaud N., Danton F., Le Jule M., Gicquel C., Le Bouc Y., Netchine I. Allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR), a powerful method for diagnosing loss of imprinting of the 11p15 region in Russell Silver and Beckwith Wiedemann syndromes. Hum. Mutat. 2011;32:249–258.
» CrossRef » Medline » Google Scholar

33. Keren B., Chantot-Bastaraud S., Brioude F., Mach C., Fonteneau E., Azzi S., Depienne C., Brice A., Netchine I., Le Bouc Y., et al. SNP arrays in Beckwith–Wiedemann syndrome: an improved diagnostic strategy. Eur. J. Med. Genet. 2013;56:546–550.
» CrossRef » Medline » Google Scholar

34. Hori N., Nakano H., Takeuchi T., Kato H., Hamaguchi S., Oshimura M., Sato K. A dyad oct-binding sequence functions as a maintenance sequence for the unmethylated state within the H19/Igf2-imprinted control region. J. Biol. Chem. 2002;277:27960–27967.
» Abstract/FREE Full Text

35. Hori N., Yamane M., Kouno K., Sato K. Induction of DNA demethylation depending on two sets of Sox2 and adjacent Oct3/4 binding sites (Sox-Oct motifs) within the mouse H19/insulin-like growth factor 2 (Igf2) imprinted control region. J. Biol. Chem. 2012;287:44006–44016.
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36. Begemann M., Spengler S., Gogiel M., Grasshoff U., Bonin M., Betz R.C., Dufke A., Spier I., Eggermann T. Clinical significance of copy number variations in the 11p15.5 imprinting control regions: new cases and review of the literature. J. Med. Genet. 2012;49:547–553.
» Abstract/FREE Full Text

37. Schoenherr C.J., Levorse J.M., Tilghman S.M. CTCF maintains differential methylation at the Igf2/H19 locus. Nat. Genet. 2003;33:66–69.
» CrossRef » Medline » Web of Science » Google Scholar

38. Engel N., Thorvaldsen J.L., Bartolomei M.S. CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. Hum. Mol. Genet. 2006;15:2945–2954.
» Abstract/FREE Full Text

39. Ideraabdullah F.Y., Abramowitz L.K., Thorvaldsen J.L., Krapp C., Wen S.C., Engel N., Bartolomei M.S. Novel cis-regulatory function in ICR-mediated imprinted repression of H19. Dev. Biol. 2011;355:349–357.
» CrossRef » Medline » Google Scholar

40. Kim Y.J., Cecchini K.R., Kim T.H. Conserved, developmentally regulated mechanism couples chromosomal looping and heterochromatin barrier activity at the homeobox gene A locus. Proc. Natl Acad. Sci. USA 2011;108:7391–7396.
» Abstract/FREE Full Text

41. Gaston V., Le Bouc Y., Soupre V., Burglen L., Donadieu J., Oro H., Audry G., Vazquez M.P., Gicquel C. Analysis of the methylation status of the KCNQ1OT and H19 genes in leukocyte DNA for the diagnosis and prognosis of Beckwith–Wiedemann syndrome. Eur. J. Hum. Genet. 2001;9:409–418.
   » CrossRef » Medline » Web of Science » Google Scholar

42. Miller S.A., Dykes D.D., Polesky H.F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215.
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