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Different mechanisms cause imprinting defects at the *IGF2/H19* locus in Beckwith–Wiedemann syndrome and Wilms' tumour

Flavia Cerrato[1,2], Angela Sparago[1,2], Gaetano Verde[1,2], Agostina De Crescenzo[1,2], Valentina Citro[3], Maria Vittoria Cubellis[3], Maria Michela Rinaldi[5], Luigi Boccuto[6], Giovanni Neri[6], Cinzia Magnani[7], Paolo D'Angelo[8], Paola Collini[9], Daniela Perotti[10], Gianfranco Sebastio[4], Eamonn R. Maher[11] and Andrea Riccio[1,*]

[1]Dipartimento di Scienze Ambientali, Seconda Università di Napoli, via Vivaldi 43, 81100 Caserta, Italy, [2]Institute of Genetics and Biophysics 'A. Buzzati Traverso', CNR, Naples, Italy, [3]Dipartimento di Biologia Strutturale e Funzionale and [4]Dipartimento di Pediatria, Università di Napoli 'Federico II', Naples, Italy, [5]U.O.C. Genetica Medica, A.O.R.N.A. Cardarelli, Naples, Italy, [6]Institute of Medical Genetics , Università Cattolica del Sacro Cuore, Rome, Italy, [7]Dipartimento Materno-Infantile, Azienda Ospedale-Università, Parma, Italy, [8]Unit of Paediatric Hematology and Oncology, 'G. Di Cristina' Children Hospital, Palermo, Italy, [9]Department of Anatomic Pathology and, [10]Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy and [11]Medical and Molecular Genetics Section, University of Birmingham, The Medical School, Birmingham, UK

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The parent of origin-dependent expression of the *IGF2* and *H19* genes is controlled by the imprinting centre 1 (IC1) consisting in a methylation-sensitive chromatin insulator. Deletions removing part of IC1 have been found in patients affected by the overgrowth- and tumour-associated Beckwith–Wiedemann syndrome (BWS). These mutations result in the hypermethylation of the remaining IC1 region, loss of *IGF2/H19* imprinting and fully penetrant BWS phenotype when maternally transmitted. We now report that 12 additional cases with IC1 hypermethylation have a similar clinical phenotype but showed neither a detectable deletion nor other mutation in the local vicinity. Likewise, no IC1 deletion was detected in 40 sporadic non-syndromic Wilms' tumours. A detailed analysis of the BWS patients showed that the hypermethylation variably affected the IC1 region and was generally mosaic. We observed that all these cases were sporadic and in at least two families affected and unaffected members shared the same maternal IC1 allele but not the abnormal maternal chromosome epigenotype. Furthermore, the chromosome with the imprinting defect derived from either the maternal grandfather or maternal grandmother. Overall, these results indicate that methylation-imprinting defects at the *IGF2–H19* locus can result from inherited mutations of the IC and have high recurrence risk or arise independently from the sequence context and generally not transmitted to the progeny. Despite these differences, the epigenetic abnormalities are usually present in the patients in the mosaic form and probably acquired by post-zygotic *de novo* methylation. Distinguishing between these two groups of cases is important for genetic counselling.

INTRODUCTION

There is increasing evidence that aberrant chromatin states leading to aberrant gene expression patterns (epimutations) have important roles in human disease (1,2). However, many characteristics of these lesions are still undefined. For instance, although it is generally accepted that epigenetic marks are cleared between generations, there are a number of cases in

*To whom correspondence should be addressed. Tel: +39 0823274599; Fax: +39 082274605; Email: andrea.riccio@unina2.it.

which this seems not to be the case. Indeed, the causes, heritability and relationship with phenotype of some epimutations have recently been the matter of debate (3–7). The human disorders caused by defects of genomic imprinting provide a paradigm for studying these issues.

Genomic imprinting is a mechanism causing the expression of a minority of genes to be monoallelic and dependent on their gametic origin (8,9). Imprinted genes are often organized in clusters and their gamete of origin-specific expression is controlled by *cis*-acting elements termed imprinting centres (ICs). These sequences are marked by different epigenetic modifications (of which DNA methylation is an essential one) on their maternal and paternal alleles. These molecular imprints are established during gametogenesis, maintained in the zygote and throughout embryo development and replaced by the new ones in the new generation of germ cells. In the somatic cells, the ICs direct by different mechanisms the parent of origin-specific expression of the imprinted genes. A 1 Mb cluster of imprinted genes lies on chromosome 11p15.5. This cluster is functionally divided into two domains that are autonomously controlled by distinct ICs [IC1 and IC2 (10,11)]. Defective expression of the 11p15.5 imprinted genes results in the Beckwith–Wiedemann syndrome (BWS, MIM 130650).

BWS is a developmental disorder characterized by variable clinical features, including overgrowth, macroglossia, abdominal wall defects and increased incidence of embryonal tumours (12). The majority of the BWS cases are sporadic. The rare familial cases show predominantly autosomal dominant inheritance and preferential expression following maternal transmission. Heterogeneous molecular defects are found in BWS (12). Only 5% of the cases (40% of the familial ones) have typical single-gene defects, consisting in loss-of-function mutations of *CDKN1C*. Approximately 20% of the cases have uniparental paternal disomy (UPD) of 11p15.5 loci, indicating that BWS is caused by excess of imprinted genes expressed from the paternal chromosome and/or defect of imprinted genes expressed from the maternal chromosome. The majority of the other cases show DNA methylation defects at either IC1 or IC2. These are accompanied by deregulation (either biallelic activation or biallelic silencing) of Domain 1 or Domain 2 imprinted genes. Microdeletions of the ICs that account for the regulatory defects have been found in some of these patients (13–17). Although many phenotypic characteristics are in common between different molecular subtypes of BWS, significant differences have been found for some of the clinical features of BWS, such as tumour risk, severity of anterior abdominal wall defect, birth weight, hemihypertrophy and ear signs (18–20).

Two genes, insulin-like growth factor 2 (*IGF2*) and *H19*, are located in Domain 1 of the 11p15.5 imprinted gene cluster. IGF2 is a paternally expressed fetal growth factor. *H19* is a maternally expressed non-coding RNA with possible tumour-suppressor functions (21). The reciprocal imprinting of *IGF2* and *H19* is controlled by IC1 in the majority of tissues. The function of this control element has been extensively studied in the mouse. IC1 [also known as *H19* differentially methylated region (DMR)] is a methylation-sensitive chromatin insulator that is located between *Igf2* and *H19* (22,23). The maternal and paternal IC1 alleles acquire differential methylation during gametogenesis. In the somatic cells, the non-methylated maternal allele interacts with the zinc-finger protein CTCF. This binding is required for preventing *de novo* methylation of the region, insulating the maternal *Igf2* promoter from downstream enhancers and activating the maternal *H19* gene. On the paternal chromosome, instead, DNA methylation prevents CTCF binding at IC1 and allows *Igf2* activation by the enhancers while the paternal *H19* promoter becomes hypermethylated and silenced. Recent evidence indicates that CTCF binding at IC1 mediates higher-order chromatin conformations in a parent of origin-specific manner (24–26). This may partition the paternal and maternal *Igf2* alleles into active and inactive chromatin domains, respectively. Five to ten percent of the patients with BWS have methylation of IC1 on both parental chromosomes. This results in biallelic activation of *IGF2* and biallelic silencing of *H19* in all tissues (27). Patients with this type of defect have higher risk of developing cancer and particularly Wilms' tumour (WT) than patients belonging to other molecular subgroups (18). Similar to BWS, patients with non-syndromic WT also have IC1 hypermethylation with *IGF2* activation and *H19* silencing, but this is restricted to cancer tissues in these cases (28).

Deletions of the IC1 region of the order of 1.4–1.8 kb have been found in BWS patients with IC1 hypermethylation (13,16,17). These cases are part of dominant inheritance pedigrees with maternal transmission. The deletions remove one to two target sequences for CTCF (CTSs) resulting in hypermethylation of the residual CTSs and co-segregate with the BWS phenotype. We found no deletion in other phenotypically similar BWS cases. In this paper, we investigate the cause of defective imprinting of *IGF2–H19* in 12 individuals with IC1 hypermethylation and no associated deletion. The results obtained suggest that these cases result from sporadic epimutations arising independently from the sequence context, probably at post-zygotic stages and have low recurrence risk. Similarly, sporadic methylation defects without accompanying IC1 deletion were found in a large series of non-syndromic WTs.

RESULTS

Search for IC1 deletions

We identified 21 individuals with the clinical features of BWS and hypermethylation of the IC1 and *H19* promoter regions as determined by Southern blotting (data not shown). Nine of these patients carried IC1 deletions and were part of five dominant pedigrees with maternal transmission (13,17). No deletion in the IC1 region was identified in the remaining 12 individuals by Southern blotting and long-range PCR (data not shown). In a previous study, the promoter and transcribed sequences of *H19* were sequenced in the DNAs of the patients BWS-16 and BWS-19 and no deletion or other mutation apart normal polymorphisms was found (29). We have now analysed the *H19* promoter region by Southern blotting and sequenced the parts of the *H19* transcribed region corresponding to known SNPs in all other patients. The normal pattern of bands detected by Southern blotting in all cases and the finding that most patients were heterozygous for at least one *H19*

intragenic polymorphic sequence variant excludes the presence of large *H19* gene and promoter deletions in most cases (data not shown). Furthermore, neither mutation of the *CDKN1C* gene apart from normal polymorphisms (30) nor methylation defects at IC2 or 11p15.5 UPD were found in these patients (data not shown). All these cases were sporadic. The clinical features of these individuals and those carrying IC1 deletions are reported in Table 1. Overall, the phenotypes of these two groups of patients are very similar, both including the characteristics more often found in the BWS patients with defect of the *IGF2-H19* imprinting domain, such as pronounced macrosomia, mild or absent defects of the abdominal wall and elevated incidence of WT (18).

Since IC1 deletions were present in 2/6 of the WTs developed in our BWS patients (Table 2), we looked for the presence of such mutations in sporadic cases of WT without other features of BWS. Consistent with the high frequency of 11p15.5 imprinting defects reported in these neoplasms, 10/40 of these tumours had IC1 hypermethylation by methylation restriction-PCR assay (MR-PCR or COBRA; Table 2). However, no IC1 deletion was detected in any of the 40 tumour cases by long-range PCR (Table 2).

Sequence analysis of IC1

The human IC1 region has a repetitive structure and contains seven target sites for CTCF (CTS) in 4 kb, with the first six CTSs arranged in two clusters of three sites each (Fig. 1A). In order to identify the cause of the epigenetic defect in the patients with IC1 hypermethylation and no deletion detected by Southern blotting, we searched for very small deletions or point mutations by sequencing the entire 4 kb region. However, no deletion was found in any of the 12 patients by this procedure. Some single-nucleotide variations with respect to the reference sequence (AF125183) were detected, but most of them corresponded to polymorphisms also found in the normal population. Indeed, common SNPs are present in three of the seven target sequences for CTCF (rs10840167, rs11042170, rs10732516). Few novel base substitutions were identified but none of them was *de novo* or present in more than one patient (Table 3). Since most of the patients were heterozygous for at least one SNP, the presence of larger deletions of the IC1 region was also excluded. It is therefore unlikely that the IC1 hypermethylation found in these patients is caused by a mutation in this region.

DNA methylation and imprinting analysis

The analysis of methylation by Southern blotting does not provide information on the methylation status of the CTSs of the IC1 region. We have previously used MR-PCR or COBRA to demonstrate that the maternal transmission of IC1 deletions is associated with hypermethylation at all the remaining CTSs and the *H19* promoter while 50% methylation is present in control individuals (17). We now used this procedure to analyse the 12 patients with hypermethylation at IC1 and no accompanying deletion (Fig. 1A). The results obtained showed differences in the extent of methylation of the individual CTSs and *H19* promoter in these patients. Methylation was analysed in the peripheral blood leukocytes and ranged from 50 to 99% at different sites and in different patients. In addition, the degree of methylation was more homogeneous among the CTSs belonging to the same cluster and the CTSs 4–7 and *H19* promoter were generally more methylated than the CTSs 1–3. Three individuals (BWS-12, BWS-19 and BWS-21) showed this characteristic in a more pronounced manner, having 50–60% methylation at the first three CTSs and 76–90% methylation at the further four CTSs and *H19* promoter. The bisulfite-sequencing analysis confirmed the contrasting methylation between the 5′ and 3′ part of IC1 in these patients (Fig. 1B). DNA derived from the skin fibroblasts was available from patient BWS-12 and the MR-PCR demonstrated 80–90% methylation at the CTSs 4–6 and 50–57% methylation at the CTSs 1–3 also in this tissue (data not shown). We also analysed IC1 and *H19* methylation at the CTSs and *H19* promoter in the parents of the patients without IC1 deletion and in nine of their unaffected sibs and found methylation levels comparable to control individuals (data not shown). We then asked whether the methylation abnormality was specific for the IC1 or was extended to other imprinted loci. In addition to the 11p15.5 IC2, which had already been found normally methylated (see above), we analysed the DMRs of *GTL2/DLK1*, *PEG3*, *ZAC* and *IGF2R* by using MR-PCR (COBRA). We found ~50% methylation at all these loci in all our patients as well as in 50 control individuals (data not shown).

We have previously reported that the BWS patients with hypermethylation and silencing of *H19* express *IGF2* from both the maternal and paternal alleles (27,31). Two of these cases were further analysed in this manuscript (BWS-16 and BWS-19) and demonstrated to have the entire IC1 region or only the CTSs 4–7 hypermethylated, respectively (Fig. 1A). We now investigated the allele-specific expression of *IGF2* and the overall expression of *H19* in the RNA derived from the skin fibroblasts of the patient BWS-12. The results demonstrated expression from both parental *IGF2* alleles, whereas expression from only the paternal allele was evident in the control, indicating loss of *IGF2* imprinting also in this case (Fig. 1C). In addition, *H19* RNA levels were 9-fold lower than in an age-matched control. No phenotypic difference was evident between the two groups of patients differing in the extension of the methylation defect at IC1. Overall, these results indicate that, in the BWS patients without IC1 deletion, the abnormal methylation extends over the entire IC1 region or affects mostly its 3′ half. All these cases, regardless of the extension of the methylation defect in the IC1, also have hypermethylation (and probably silencing) of *H19* and in several of them *IGF2* has been demonstrated to be activated on both parental alleles. Furthermore, the methylation defect is specific for the 11p15.5 IC1 locus, is generally incomplete suggesting epigenetic mosaicism, and is not present in the unaffected relatives.

Segregation of 11p15.5 haplotypes

Since BWS results from an imprinting defect on the maternal chromosome, we analysed the segregation of the maternal IC1 allele of the index patient in the families without IC1 microdeletion after construction of 11p15.5 haplotypes by

Table 1. Clinical features of the BWS patients with IC1 hypermethylation

Clinical features	IC1 hypermethylation without microdeletion	IC1 hypermethylation with microdeletion
Macrosomia (weight perc. >90)	9/11	7/8
Polydramnios	2/11	2/8
Macroglossia	9/11	7/8
Rectum diastasis	6/11	5/8
Umbilical hernia	6/11	3/8
Exomphalos	0/11	0/8
Inguinal hernia	1/11	0/8
Hemihypertrophy	3/11	4/8
Facial asymmetry	2/11	2/8
Hepato-/spleno-megaly	7/11	4/8
Neonatal hypoglycaemia	6/11	2/8
Ear pits/creases	4/11	2/8
Facial dysmorphology	4/11	3/8
Naevus flammeous	2/11	3/8
Renal abnormalities	6/11	4/8
WT[a]	2/11 (2/9)	2/8 (2/6)
Ureteral abnormalities	1/11	2/8
VSD/ASD/PDA shunt	1/11	0/8
Convulsions	1/11	0/8
Mental retardation	0/11	1/8

[a]Incidence at >6 years is shown in parenthesis.

Table 2. Molecular defects at 11p15.5 in syndromic and non-syndromic WTs

Molecular defect	Non BWS-Wilms ($n = 40$)	BWS Wilms ($n = 6$)
IC1 hypermethylation with microdeletion	0	2
IC1 hypermethylation without microdeletion	10	2
Loss of maternal allele (UPD or LOH)	18	2
No defect	12	0

microsatellite analysis (Fig. 2A). In one family (BWS-20), the affected and an unaffected sib inherited the same 11p15.5 haplotype from their mother. In three additional families (BWS-11, BWS-17 and BWS-21), different 11p15.5 haplotypes were transmitted to the patients and their healthy siblings from their mothers. However, in family BWS-11, the propositus and her healthy mother shared the same 11p15.5 haplotype on the maternal chromosome. So, in at least two families, affected and non-affected individuals had the same maternal IC1 allele.

The grandparental origin of the chromosomes carrying the imprinting defect was determined by microsatellite analysis (Fig. 2A) or (if grandparents were not available) DNA methylation/SNP test (Fig. 2B). In this procedure, the grandpaternal and grandmaternal IC1 alleles were distinguished in the DNA of the mothers because of their differential methylation (which was always all-or-none on the two parental alleles) determined by bisulfite sequencing. In case of heterozygosity, SNPs were used to determine which maternal allele was transmitted to the probands. Overall, the results showed that in five informative cases the chromosome carrying the imprinting alteration derived from the maternal grandfather, whereas in one case derived from the maternal grandmother (Table 4).

Mutation analysis of CTCF

Since CTCF binding is required for preventing *de novo* methylation at IC1 (32–35), we looked for mutations at the *CTCF* locus in the BWS patients with IC1 hypermethylation and no accompanying defect in *cis*. We sequenced all exons and flanking intronic regions in six of such patients. Only a single-nucleotide variation with respect to the reference sequence (NT_010498, nucl. 21210663–21287287) was found in the 5′-UTR (Ex1 C/G19) of one individual (BWS-10) and an SNP also present in the normal population was found in the 3′-UTR (rs6499137) of another individual (BWS-12). No change in the coding sequence was present in any of these patients. Hemizygosity at the *CTCF* locus was also excluded in all cases by analysing a microsatellite present in intron 7 (rs3223529). Therefore, these results indicate that *CTCF* gene mutations are not a common cause of IC1 hypermethylation in BWS.

DISCUSSION

Gain of methylation defects at IC1 are found in only 5–10% of the BWS cases (12,18). However, the high incidence of WT associated with these molecular abnormalities makes them particularly important to study. Some of the patients with this type of abnormal methylation have been demonstrated to have germline deletions in the IC1 region and high recurrence risk (13,17). Other BWS patients with similar epigenetic defect at IC1 had no associated deletion. It was unclear whether these patients had any other mutation in *cis*, what was the nature of their molecular defect and what was their recurrence risk. By analysing 12 patients

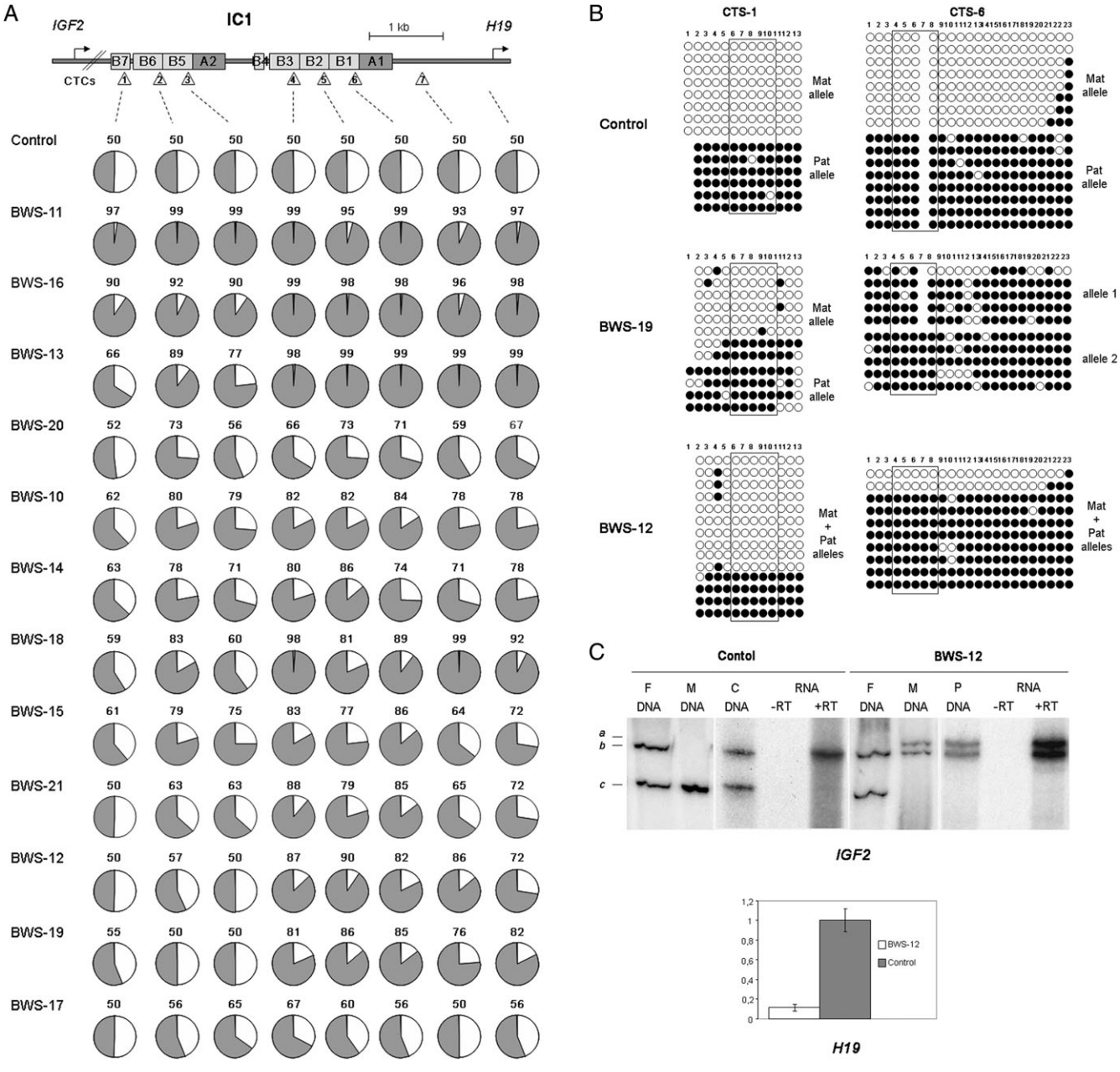

Figure 1. Methylation of the CTSs and *H19* promoter and *IGF2* and *H19* expression in the BWS patients with IC1 hypermethylation and no associated deletion.(**A**) Summary of the results obtained on 12 BWS patients and 40 control individuals. DNA methylation at CpGs included in the CTSs and *H19* promoter was assayed by MR-PCR (COBRA) in leukocyte DNA. The average methylation levels detected in control individuals ($50 \pm 5\%$) are shown for comparison. The extent of methylation at each CpG site is indicated with pie charts filled in black. Each sample was run in duplicate. SD was $\sim$5%. A diagram showing the structure of the human IC1 region is present in the upper part of the figure. A- and B-type repeats are indicated by boxes of different colours, whereas the CTSs are indicated by triangles. (**B**) DNA methylation profiles of CTCF site 1 and CTCF site 6 and flanking regions in patients BWS-19 and BWS-12 and a control individual, as determined by bisulfite-genomic sequencing. Each line corresponds to a single template DNA molecule cloned; each circle corresponds to a CpG dinucleotide. Filled circles designate the methylated cytosines; open circles, unmethylated cytosines. The CpGs included in the CTCF sites are framed. When indicated, the parental alleles were discriminated by SNPs. (**C**) Analysis of *IGF2* and *H19* expression in patient BWS-12. RNAs extracted from cultured skin fibroblasts of the patient and a control individual were analysed by reverse transcriptase–PCR. In the upper panel, the parental alleles of *IGF2* were discriminated by typing for a direct repeat polymorphism (Ca_n repeat) present in the 3′-untraslated region of the *IGF2* gene. Three different alleles were observed. Both the maternal and paternal *IGF2* alleles (*a* and *b*) were expressed in the fibroblasts of the patient, but only the paternal allele (*b*) was expressed in the control. To exclude contamination from DNA, the RNAs were amplified with (RT+) and without (RT−) previous treatment with reverse transcriptase. In the lower panel, the overall *H19* RNA was determined by quantitative real-time RT–PCR using primers specific for the *H19* transcript and the *GAPD* mRNA. Note that in the BWS patients without IC1 deletion the hypermethylation is generally mosaic and affects predominantly the CTSs that are closer to the *H19* promoter. Regardless of the extension of the IC1 methylation defect, all these cases also have hypermethylation of *H19* and several of them have been demonstrated to express *IGF2* from both parental alleles and have *H19* silenced.

Table 3. Sequence variations of the IC1 region

Patient	Variation	Position (accession no. AF125183)
BWS-10	A/G	7679
BWS-12	T/C[a]	5198
BWS-14	C/T	4936
BWS-18	T/C	6653
BWS-19	G/A	4613
	G/A	5906

[a]T5198>C was present in homozygosity in this individual.

Table 4. Grandparental origin of the chromosome carrying the imprinting defect

Origin	IC1 microdeletion	No IC1 microdeletion
Maternal grandmother	1	1
Maternal grandfather	8[a]	5

[a]In one patient, the deletion is *de novo* or due to germline mosaicism.

Table 5. PCR primers used for the sequence analysis

H19	Forward primer	Reverse primer
Ex1	5′-tggcggggagaagcagacac-3′	5′-gcgtcaccaagtccactgtg– 3′
Ex 4/5	5′-ttcctgccacacttggggtg-3′	5′-tgatgtggtggctggtggtc-3′
Ex 5	5′-ccaccagccaccacatcatc-3′	5′-gaaggctgctccgtgatgtc-3′
CDKN1C	Forward primer	Reverse primer
Promoter	5′-gcgccctttaactcccgccc-3′	5′-gctagctcgctcgctcaggc-3′
5′-UTR	5′-gggctgggcgttccacaggc-3′	5′-gatgtgctgcggagggacgc-3′
3′-UTR	5′-ctcggctgggaccgttcatg-3′	5′-agctagtgcgtggcagagcg-3′
CTCF	Forward primer	Reverse primer
Ex1	5′-cgtcccttcccttatcagcac-3′	5′-cccatcgtgacacctagaggc-3′
Ex2	5′-atggctcggttagtgacatg-3′	5′-gccaacaaatcctccaactc-3′
Ex3	5′-gcctaattcattcaccaaag-3′	5′-cccctgaagttcttctactg-3′
Ex3	5′-atatggaggaacagcccataaac-3′	5′-caacttgcatatgcactgtgttg-3′
Ex4	5′-aacactttgaaactctgcagcaag-3′	5′-cgctggagtcagcttgaatg-3′
Ex5	5′-catagcagttctgtgccacac-3′	5′-tcatcttaagtccgtttgggtag-3′
Ex6	5′-actgtgctcttgttacagtctgtg-3′	5′-aacaatggggacacacagtg-3′
Ex7	5′-gtgtagcatatctgccacctg-3′	5′-tgttatgagagtcagaaggtgaag-3′
Ex8	5′-gaatcgagaaatgtattagtaacttg-3′	5′-gtgacattcctcataatccacag-3′
Ex9	5′-aatacctgttggccacatgc-3′	5′-accatgctctgcagaggaag-3′
Ex10	5′-gagcctcccccttagagaacc-3′	5′-gcctctatatcccagcatatcc-3′
Ex11	5′-ttcatcttccaccacccttctc-3′	5′-gacttcctcagatgttcctcagt-3′
Ex12	5′-cattgctgacatcccgttc-3′	5′-gaccataccagaaactttgcc-3′
Ex12	5′-gcttggagtcagctgcaca-3′	5′-cagcctggaaccatgtatgc-3′

with IC1 hypermethylation and no deletion, we found no mutation in the critical IC1 elements and demonstrated that, in at least two cases, the maternal IC1 allele of the index patient segregated in one of his healthy relatives. A detailed methylation analysis showed that the hypermethylation was extended over the entire or only the 3′ half of the IC1 region, did not affect other imprinted loci, generally occurred in the mosaic form and was never present in the unaffected relatives. We also observed that the chromosome carrying the imprinting abnormality derived from either the maternal grandfather or maternal grandmother. These results indicate that, in the absence of deletions, IC1 hypermethylation generally occurs as sporadic epimutation and is associated with low recurrence risk. Consistent with this conclusion, no deletion was detected in a large series of non-syndromic sporadic WTs.

In the mouse, the CTSs have been shown to be required for the post-zygotic maintenance of the methylation-free status of the maternal IC1 allele (32–35). Consistent with these observations, deletions removing one to two CTSs of the human IC1 result in the hypermethylation of its maternal allele (13,17). However, we found no mutation in any of the seven CTSs of another 12 BWS patients with IC1 hypermethylation. In addition, the segregation of the chromosome with the imprinting defect in the healthy relatives excludes inherited mutations in the entire chromosome 11p15.5 region of two individuals. Therefore, although we cannot rule out the presence of *de novo* mutations at sites distant from IC1, our results strongly support the hypothesis that in a subset of BWS patients IC1 epimutations occurr independently of the sequence context.

We previously observed that all IC1 CTSs are completely and exclusively methylated on the paternal chromosome in normal leukocyte DNA, whereas incomplete hypermethylation of the maternal allele is present in the BWS patients with 1.4–1.8 kb IC1deletions (17). We now found incomplete methylation at IC1 also in the majority of patients without deletions suggesting that this imprinting defect is generally present in the mosaic form. Mosaicism can in part explain the high variability of the clinical phenotype that is characteristic of this disorder. In addition, diagnostic problems may be encountered with individuals who may not have abnormal methylation in their leukocyte DNA. To avoid these problems, it may be useful in the future to analyse DNAs derived from more than one tissue (e.g. blood leukocytes and buccal mucosa).

Imprinting defects at ICs can derive from failure of erasure, establishment or maintenance of the parental marks (8). One way to approach this problem is to investigate the grandparental origin of the chromosome with the imprinting defect (36). We found that in the BWS patients the chromosome with abnormal IC1 methylation derived from either the maternal grandfather or the maternal grandmother. Since the majority of these cases also showed mosaic hypermethylation, it is likely that the methylation defect was acquired after erasure of the parental epigenetic marks and probably at a post-zygotic stage as result of defective protection from *de novo* methylation of the maternal IC1 allele. A similar mechanism is also likely to be involved in the cases with IC1 deletion (17). However, an incomplete imprint erasure resulting in an unstable methylation is also possible, at least in some cases.

The ultimate effect of IC1 methylation on the maternal chromosome is *H19* silencing (by methylation spreading into the promoter region) and *IGF2* activation [by inactivation of the insulator activity (8)]. We found that in some of the BWS patients hypermethylation is more evident on the four CTSs that are located closer to the *H19* promoter. This may indicate that methylation of the CTSs 4–7 is sufficient to cause silencing of *H19* and/or activation of *IGF2*. Indeed, gain of methylation at the *H19* promoter was observed in all the patients regardless of the extension of IC1 hypermethylation and biallelic activation of *IGF2* could be demonstrated in two of the patients with limited IC1 methylation.

Table 6. MR-PCR conditions for the methylation analysis of the DMRs at *ZAC*, *IGF2R*, *PEG3* and *GTL2/DLK1* loci

Locus	PCR primers	Annealing temperature	$MgCl_2$	PCR product size	Enzyme
ZAC	F: 5′-gtgtgggtgtygtttagttttttt-3′ R: 5′-aactaaataacaaataacaaatacc-3′	55°C	2 mM	224 bp	*Taq*I
IGF2R	F: 5′-tatgttggggataggttttgggag-3′ R: 5′-taaaataacccctctatatcaaaaacccc-3′	55°C	1.5 mM	330 bp	*Bst*UI
PEG3	F: 5′-tattttagtgggtggggtttgagtag-3′ R: 5′-ccctaaacctcctaaactaaatctaaaaac-3′	57°C	1.5 mM	322 bp	*Bst*UI
GTL2/DLK1	F: 5′-ttaggttggaattgttaagagtttgtggatt-3′ R: 5′-ataaactacactactaaaaactacatttaaa-3′	60°C	1.5 mM	384 bp	*Taq*I

Cooperative interaction between proteins bound to adjacent CTSs (33) may explain the more homogeneous methylation inside each cluster of CTSs.

Since we did not find a mutation in *cis*, it is possible that a regulatory *trans*-acting factor is defective in the BWS cases with IC1 hypermethylation and no deletion. Knocking-down of *CTCF* in mouse oocytes and in cultured cells results in gain of methylation of IC1 (7,37). We ruled out the presence of a mutation of the CTCF gene in our patients. It cannot be excluded, however, that a mutation is present in other modifier genes. Considering the sporadic nature of these cases, the possibility that IC1 hypermethylation occurs as consequence of stochastic events or environmental influence should also be envisaged (38).

The 11p15.5 imprinted gene cluster is frequently affected in WTs (28). Either maternal deletion/paternal duplication (LOH) or IC1 hypermethylation coupled to *H19* silencing and *IGF2* activation (LOI) can be found in a high proportion of tumours (see also Table 2). In addition, the individuals who have soma-wide IC1 hypermethylation or 11p15.5 paternal UPD represent the molecular subgroup of BWS patients showing the highest risk of developing WT (18–20). Consistent with these observations, we found that 2/6 BWS patients with IC1 deletion had developed this neoplasm by 6 years of age. However, no IC1 deletion was found in 40 sporadic non-syndromic WTs enrolled by Pediatric Oncology Units affiliated to Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP). A previous American study demonstrated the absence of point mutations in the IC1 sequence of a series of sporadic WTs (39). Overall, these data suggest that IC1 imprinting defects are generally not caused by a mutation in *cis* in non-syndromic WT as well as sporadic BWS.

Although the common hallmark and probably the ultimate cause of the imprinting defects at the *IGF2/H19* locus is represented by hypermethylation of IC1, our studies demonstrate that this epigenetic abnormality can result from more than one mechanism in BWS and WT. In a first group of patients, we found that the epimutation is a direct consequence of a mutation *in cis*, consisting of a deletion of one to two CTSs. In these cases, the methylation defect and disease phenotype are reproduced whenever the mutation is transmitted through the maternal germline. In another group of patients, who carry no IC1 deletion, the epimutation is independent of the local DNA sequence and generally not transmitted to the progeny. Sporadic BWS and non-syndromic WTs belong to the second group of cases. Despite these differences, the IC1 epimutation is generally present in the patients in the mosaic form and probably acquired by postzygotic *de novo* methylation, providing an example of how intricate the relationship between genotype and epigenotype can be.

MATERIALS AND METHODS

Patients

BWS was diagnosed in all patients according to the criteria described in the literature (http://www.geneclinics.org). The clinical features of the BWS patients included in this study are summarized in Table 1. The study included 40 WT patients enrolled in the AIEOP 2003 WT trial. In 37 of these cases, there was no evidence of associated congenital abnormalities. The three other cases were associated with WAGR, Turner and Down syndrome phenotypes, respectively.

All the genetic analyses were performed after the informed consent was obtained from the parents of the patients. The experimental plan was approved by the ethical committees of the Second University of Naples and Istituto Nazionale Tumori, INT, Milan.

Southern blot, PCR, DNA sequencing and microsatellite analyses

Southern blot hybridization was performed on DNA purified from blood leukocytes as described (13,31). PCR amplification of the *H19* DMR was obtained from leukocyte DNA by using the primers 5′-AGAGATGGGATTTCGTCAGGTTG G-3′ and 5′- CATTTCCGTCTCCACAGCCACAAC-3′ and the *Taq* BIO-X-ACT Long (BIOLINE) as described (13). The fragments generated were gel-purified and cloned into pCR II (Invitrogen). The primers used for sequencing the *H19* and *CTCF* gene and the promoter, 5′-UTR and 3′-UTR of *CDKN1C* are reported in Table 5. The primers used for sequencing *CDKN1C* exons 3–4 were previously described (40). DNA sequencing was obtained from PRIMM (Italy). UPD and LOH at 11p15.5 loci were determined by microsatellite analysis, as described (18).

DNA methylation analyses

The DNA methylation of IC1 (*H19* DMR) and *H19* promoter was analysed by *Hpa*II digestion and Southern blot hybridization, sodium bisulfite sequencing and bisulfite treatment coupled with restriction enzyme digestion (MR-PCR), as described (13). The methylation of IC2 (KvDMR1) was

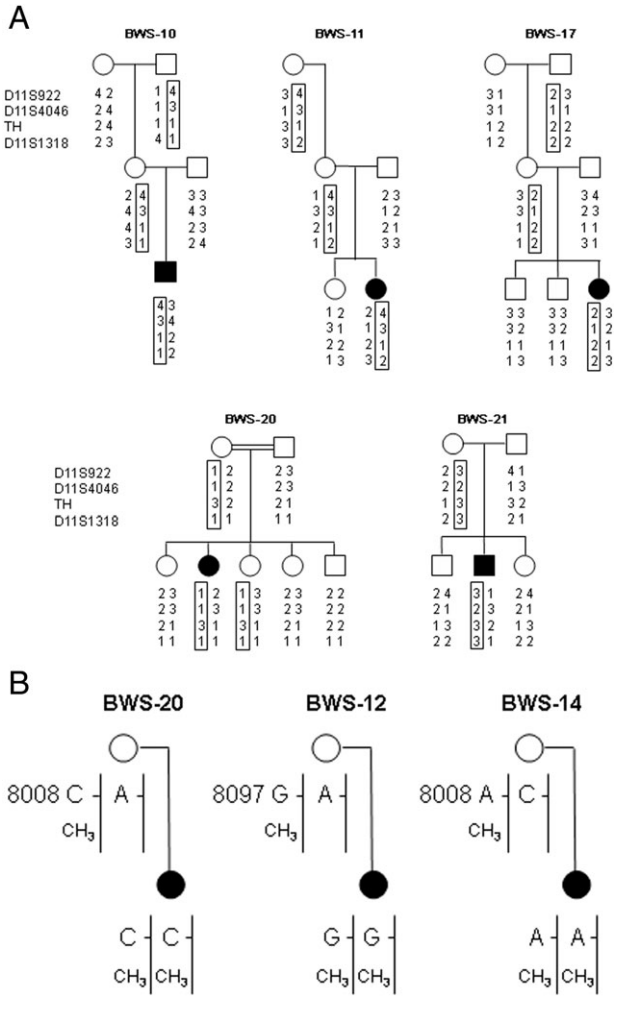

Figure 2. Segregation of chromosome 11p15.5 genotypes in BWS families with IC1 imprinting defect and no associated deletion. (**A**) Construction of haplotypes by microsatellite analysis. The haplotype associated with the imprinting defect in the patient is framed. The chromosome location of the IC1 region is between *TH* and D11S1318. (**B**) Determination of the grandparental origin of the chromosome with the imprinting defect by combined DNA methylation/SNP test. The methylation of the IC1 region was analysed by bisulfite sequencing in the mothers of patients BWS-20, BWS-12 and BWS-14. Alleles were discriminated by SNPs and parental origin was assessed by assuming that the paternal IC1 allele was methylated and the maternal IC1 allele was not in normal individuals. DNA sequencing allowed to determine which maternal allele was transmitted to the probands. The SNPs and their positions in the AF125183 sequence are indicated. Note that affected and unaffected individuals share the same IC1 allele on the maternally inherited chromosome in some families and the chromosome with the imprinting defect in the probands derive from either the maternal grandfather or maternal grandmother.

analysed by *Not*I digestion and Southern blotting hybridization, as described (18). The DNA methylation of the DMRs of *ZAC*, *IGF2R*, *PEG3* and *GTL2/DLK1* were analysed by MR-PCR. The PCR conditions and the enzymes used for each DMR are described in Table 6.

Gene expression analysis

The allele-specific expression of *IGF2* and the overall expression of *H19* were analysed as previously described (13).

ELECTRONIC-DATABASE INFORMATION

URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim [for BWS (MIM 130650)]. http://www.mgu.har.mrc.ac.uk/research/imprinting/;http://igc.otago.ac.nz/home.html; http://www.geneclinics.org

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Conflict of Interest statement. None declared.

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REFERENCES

1. Horsthemke, B. (2006) Epimutations in human disease. *Curr. Top. Microbiol. Immunol.*, **310**, 45–59.
2. Feinberg, A.P. (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature*, **447**, 433–440.
3. Suter, C.M. and Martin, D.I. (2007) Inherited epimutation or a haplotypic basis for the propensity to silence? *Nat. Genet*, **39**, 573.
4. Suter, C.M. and Martin, D.I. (2007) Reply to 'Heritable germline epimutation is not the same as transgenerational epigenetic inheritance'. *Nat. Genet*, **39**, 575–576.
5. Horsthemke, B. (2007) Heritable germline epimutations in humans. *Nat. Genet.*, **39**, 573–574.
6. Chong, S., Youngson, N.A. and Whitelaw, E. (2007) Heritable germline epimutation is not the same as transgenerational epigenetic inheritance. *Nat. Genet.*, **39**, 574–575.
7. Leung, S.Y., Chan, T.L. and Yuen, S.T. (2007) Reply to 'Heritable germline epimutation is not the same as transgenerational epigenetic inheritance'. *Nat. Genet.*, **39**, 576.
8. Reik, W. and Walter, J. (2001) Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet.*, **2**, 21–32.
9. Edwards, C.A. and Ferguson-Smith, A.C. (2007) Mechanisms regulating imprinted genes in clusters. *Curr. Opin. Cell Biol.*, **19**, 281–289.
10. Ainscough, J.F.X., Koide, T., Tada, M., Barton, S. and Surani, M.A. (1997) Imprinting of Igf2 and H19 from a 130 kb YAC transgene. *Development*, **124**, 3621–3632.
11. Cerrato, F., Sparago, A., Di Matteo, I., Zou, X., Dean, W., Sasaki, H., Smith, P., Genesio, R., Bruggemann, M., Reik, W. and Riccio, A. (2005) The two-domain hypothesis in Beckwith–Wiedemann syndrome: autonomous imprinting of the telomeric domain of the distal chromosome 7 cluster. *Hum. Mol. Genet.*, **14**, 503–511.
12. Weksberg, R., Shuman, C. and Smith, A.C. (2005) Beckwith–Wiedemann syndrome. *Am. J. Med. Genet. C Semin. Med. Genet.*, **137**, 12–23.
13. Sparago, A., Cerrato, F., Vernucci, M., Ferrero, G.B., Cirillo Silengo, M. and Riccio, A. (2004) Microdeletions in the human H19 DMR result in loss of IG F2 imprinting and Beckwith–Wiedemann. *Nat. Genet.*, **36**, 958–960.

14. Niemitz, E.L., DeBaun, M.R., Fallon, J., Murakami, K., Kugoh, H., Oshimura, M. and Feinberg, A.P. (2004) Microdeletion of LIT1 in familial Beckwith–Wiedemann syndrome. *Am. J. Hum. Genet.*, **75**, 844–849.
15. Prawitt, D., Enklaar, T., Gartner-Rupprecht, B., Spangenberg, C., Oswald, M., Lausch, E., Schmidtke, P., Reutzel, D., Fees, S., Lucito, R. *et al.* (2005) Microdeletion of target sites for insulator protein CTCF in a chromosome 11p15 imprinting center in Beckwith–Wiedemann syndrome and Wilms' tumour. *Proc. Natl Acad. Sci. USA*, **102**, 4085–4090.
16. Cerrato, F., Sparago, A., Farina, L., Ferrero, G.B., Cirillo Silengo, M. and Riccio, A. (2005) Reply to Microdeletions in the human *H19* DMR result in loss of *IGF2* imprinting and Beckwith–Wiedemann. *Nat. Genet.*, **37**, 786–787.
17. Sparago, A., Russo, S., Cerrato, F., Ferraiuolo, S., Castorina, P., Selicorni, A., Schwienbacher, C., Negrini, M., Ferrero, G.B., Silengo, M.C. *et al.* (2007) Mechanisms causing imprinting defects in familial Beckwith–Wiedemann syndrome with Wilms' tumour. *Hum. Mol. Genet.*, **16**, 254–264.
18. Cooper, W.N., Luharia, A., Evans, G.A., Raza, H., Haire, A.C., Grundy, R., Bowdin, S.C., Riccio, A., Sebastio, G., Bliek, J. *et al.* (2005) Molecular subtypes and phenotypic expression of Beckwith–Wiedemann syndrome. *Eur. J. Hum. Genet.*, **13**, 1025–1032.
19. DeBaun, M.R., Niemitz, E.L., McNeil, D.E., Brandenburg, S.A., Lee, M.P. and Feinberg, A.P. (2002) Epigenetic alterations of H19 and LIT1 distinguish patients with Beckwith–Wiedemann syndrome with cancer and birth defects. *Am. J. Hum. Genet.*, **70**, 604–611.
20. Weksberg, R., Nishikawa, J., Caluseriu, O., Fei, Y.L., Shuman, C., Wei, C., Steele, L., Cameron, J., Smith, A., Ambus, I. *et al.* (2001) Tumor development in the Beckwith–Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. *Hum. Mol. Genet.*, **10**, 2989–3000.
21. Hao, Y., Crenshaw, T., Moulton, T., Newcomb, E. and Tycko, B. (1993) Tumour-suppressor activity of H19 RNA. *Nature*, **365**, 764–767.
22. Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M. and Tilghman, S.M. (2000) CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature*, **405**, 486–489.
23. Bell, A.C. and Felsenfeld, G. (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature*, **405**, 482–485.
24. Kurukuti, S., Tiwari, V.K., Tavoosidana, G., Pugacheva, E., Murrell, A., Zhao, Z., Lobanenkov, V., Reik, W. and Ohlsson, R. (2006) 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*, **103**, 10684–10689.
25. Ling, J.Q., Li, T., Hu, J.F., Vu, T.H., Chen, H.L., Qiu, X.W., Cherry, A.M. and Hoffman, A.R. (2006) CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. *Science*, **312**, 269–272.
26. Murrell, A., Heeson, S. and Reik, W. (2004) Interaction between differentially methylated regions partitions the imprinted genes *Igf2* and *H19* into parent-specific chromatin loops. *Nat. Genet.*, **36**, 889–893.
27. Joyce, J.A., Lam, W.K., Catchpoole, D.J., Jenks, P., Reik, W., Maher, E.R. and Schofield, P.N. (1997) Imprinting of IGF2 and H19: lack of reciprocity in sporadic Beckwith–Wiedemann syndrome. *Hum. Mol. Genet.*, **6**, 1543–1548.
28. Feinberg, A.P., Cui, H. and Ohlsson, R. (2002) DNA methylation and genomic imprinting: insights from cancer into epigenetic mechanisms. *Semin. Cancer Biol.*, **12**, 389–398.
29. Catchpoole, D., Smallwood, A.V., Joyce, J.A., Murrell, A., Lam, W., Tang, T., Munroe, D., Reik, W., Schofield, P.N. and Maher, E.R. (2000) Mutation analysis of H19 and NAP1L4 (hNAP2) candidate genes and IGF2 DMR2 in Beckwith–Wiedemann syndrome. *J. Med. Genet.*, **37**, 212–215.
30. Tokino, T., Urano, T., Furuhata, T., Matsushima, M., Miyatsu, T., Sasaki, S. and Nakamura, Y. (1996) Characterization of the human p57KIP2 gene: alternative splicing, insertion/deletion polymorphisms in VNTR sequences in the coding region, and mutational analysis. *Hum. Genet.*, **97**, 625–631.
31. Reik, W., Brown, K.W., Schneid, H., Le Bouc, Y., Bickmore, W. and Maher, E.R. (1995) Imprinting mutations in the Beckwith–Wiedemann syndrome suggested by altered imprinting pattern in the IGF2–H19 domain. *Hum. Mol. Genet.*, **4**, 2379–2385.
32. Schoenherr, C.J., Levorse, J.M. and Tilghman, S.M. (2002) CTCF maintains differential methylation at the Igf2/H19 locus. *Nat. Genet*, **33**, 66–69.
33. Pant, V., Kurukuti, S., Pugacheva, E., Shamsuddin, S., Mariano, P., Renkawitz, R., Klenova, E., Lobanenkov, V. and Ohlsson, R. (2004) Mutation of a single CTCF target site within the *H19* imprinting control region leads to loss of *Igf2* imprinting and complex patterns of *de novo* methylation upon maternal inheritance. *Mol. Cell. Biol.*, **24**, 3497–3504.
34. Szabo, P.E., Tang, S.H., Silva, F.J., Tsark, W.M. and Mann, J.R. (2004) Role of CTCF binding sites in the Igf2/H19 imprinting control region. *Mol. Cell. Biol.*, **24**, 4791–4800.
35. Engel, N., Thorvaldsen, J.L. and Bartolomei, M.S. (2006) CTCF binding sites promote transcription initiation and prevent DNA methylation on the maternal allele at the imprinted H19/Igf2 locus. *Hum. Mol. Genet.*, **15**, 2945–2954.
36. Buiting, K., Gross, S., Lich, C., Gillessen-Kaesbach, G., el-Maarri, O. and Horsthemke, B. (2003) Epimutations in Prader–Willi and Angelman syndromes: a molecular study of 136 patients with an imprinting defect. *Am. J. Hum. Genet.*, **72**, 571–577.
37. Fedoriw, A.M., Stein, P., Svoboda, P., Schultz, R.M. and Bartolomei, M.S. (2004) Transgenic RNAi reveals essential function for CTCF in H19 gene imprinting. *Science*, **303**, 238–240.
38. Jaenisch, R. and Bird, A. (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.*, **33** (suppl.), 245–254.
39. Cui, H., Niemitz, E.L., Ravenel, J.D., Onyango, P., Brandenburg, S.A., Lobanenkov, V.V. and Feinberg, A.P. (2001) Loss of imprinting of insulin-like growth factor-II in Wilms' tumour commonly involves altered methylation but not mutations of CTCF or its binding site. *Cancer Res.*, **61**, 4947–4950.
40. Lew, J.M., Fei, Y.L., Aleck, K., Blencowe, B.J., Weksberg, R. and Sadowski, P.D. (2004) CDKN1C mutation in Wiedemann–Beckwith syndrome patients reduces RNA splicing efficiency and identifies a splicing enhancer. *Am. J. Med. Genet. A*, **127**, 268–276.