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Mechanisms causing imprinting defects in familial Beckwith–Wiedemann syndrome with Wilms' tumour

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The imprinted expression of the *IGF2* and *H19* genes is controlled by the Imprinting Centre 1 (IC1) at chromosome 11p15.5. This is a methylation-sensitive chromatin insulator that works by binding the zinc-finger protein CTCF in a parent-specific manner. Microdeletions abolishing some of the CTCF target sites (CTSs) of IC1 have been associated with the Beckwith–Wiedemann syndrome (BWS). However, the link between these mutations and the molecular and clinical phenotypes was debated. We have identified two novel families with IC1 deletions, in which individuals with the clinical features of the BWS are present in multiple generations. By analysing the methylation pattern at the *IGF2-H19* locus together with the clinical phenotypes in the individuals with maternal and those with paternal transmission of five different deletions, we demonstrate that maternal transmission of 1.4–1.8 kb deletions in the IC1 region co-segregates with the hypermethylation of the residual CTSs and BWS phenotype with complete penetrance, whereas normal phenotype is observed upon paternal transmission. Although gene expression could not be assayed in all cases, the methylation detected at the *IGF2* DMR2 and *H19* promoter suggests that IC1 hypermethylation is consistently associated with biallelic activation of *IGF2* and biallelic silencing of *H19*. Comparison of these deletions with a 2.2 kb one previously reported by another group indicates that the spacing of the CTSs on the deleted allele is critical for the gain of the abnormal methylation and penetrance of the clinical phenotype. Furthermore, we observe that the hypermethylation resulting from the deletions is always mosaic, suggesting that the epigenetic defect at the *IGF2-H19* locus is established post-zygotically and may cause body asymmetry and heterogeneity of the clinical phenotype. Finally, the IC1 microdeletions are associated with a high incidence of Wilms' tumour, making their molecular diagnosis particularly important for genetic counselling and tumour surveillance at follow-up.

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INTRODUCTION

Genomic imprinting is an epigenetic mechanism causing the expression of a minority of genes to be monoallelic and dependent on its gametic origin (1–3). Correct imprinting is required for normal development, whereas defective imprinting is associated with human disease (4). Beckwith–Wiedemann syndrome (BWS) (MIM 130650) is a developmental disorder characterized by variable clinical features, including overgrowth, macroglossia, abdominal wall defects and increased incidence of embryonal tumours that are caused by defective expression of imprinted genes located on chromosome 11p15.5 (5–7). At this locus, a 1 Mb cluster of imprinted genes is present. The cluster is functionally divided into two domains that are autonomously controlled by separate Imprinting Control Regions or Imprinting Centres (IC1 and IC2) (8,9). These are CpG-rich regions that work under different mechanisms, but share as a common feature to be differentially methylated on the maternally and paternally derived chromosomes [differentially methylated regions (DMRs)].

BWS occurs with a frequency of 1/13 700. The majority of the BWS cases are sporadic. The less frequent (10–15%) familial cases show heterogeneous modes of inheritance (10). Several types of molecular defects are found in BWS (6). Only 5% of the cases (40% of the familial ones) have single-gene defects, consisting of loss-of-function mutations of *CDKN1C*. A minority of patients have chromosome aberrations, consisting in 11p15.5 paternal trisomy (11) or balanced maternal translocations with breakpoint at 11p15.5. Another 20% of the cases have uniparental paternal disomy (UPD) of 11p15.5 loci, indicating that BWS is caused by the excess of imprinted genes expressed from the paternal chromosome and/or defect of imprinted genes expressed from the maternal chromosome. Inherited deletions of the ICs have been recently reported in a small number of patients with BWS (12–14). No mutation but abnormal methylation at IC1 or IC2 has been found in the majority of the other cases so far (6).

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 gene with an important role in cancer development (15). *H19* is a maternally expressed non-coding RNA with possible tumour-suppressor functions (16). 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* DMR) is a methylation-sensitive chromatin insulator located between *IGF2* and *H19* (17,18). Its non-methylated maternal allele interacts with the multi-zinc-finger protein CTCF. This binding is required on the maternal chromosome for maintaining the non-methylated status of the region and for preventing the activation of the *IGF2* promoter by downstream enhancers that activate the *H19* gene instead. On the paternal chromosome, conversely, DNA methylation prevents CTCF binding at IC1 and allows the enhancer-mediated activation of *IGF2*, whereas the *H19* promoter is hypermethylated and silenced. Recent evidence indicates that the methylation-sensitive binding of CTCF at IC1 mediates higher order chromatin conformations in a parent of origin-specific manner (19,20). In particular, the two parental IC1 alleles interact with two different DMRs of *Igf2*: DMR1 on the maternal chromosome and DMR2 on the paternal chromosome (21). This may partition the maternal and paternal *Igf2* alleles into inactive and active chromatin domains, respectively. About 10% of the patients with BWS have gain of methylation on the maternal IC1 and, therefore, display methylation of IC1 on both parental chromosomes. This results in biallelic activation of *IGF2* and biallelic silencing of *H19*. Patients with such type of defect have higher risk of developing cancer and Wilms' tumour, particularly than those belonging to other molecular subgroups (22).

Microdeletions of IC1 have been associated with BWS (12,14). However, the consequence of these mutations on the molecular and clinical phenotypes is a matter of controversy (23,24). The human IC1 has a repetitive structure and overall contains seven target sites for CTCF (CTS). We have previously described three cases in which a maternally inherited deletion causing the loss of one or two CTSs was accompanied by gain of methylation of the remaining CTSs, disruption of *IGF2* and *H19* imprinting and disease phenotype (12,24). Prawitt *et al*. (14) described a larger deletion eliminating three CTSs of the maternal IC1 that was associated with loss of *IGF2* imprinting but did not alter methylation of the sequences flanking the deletion and was present in both affected and unaffected individuals. Indeed, an additional mutation consisting in duplication of the 11p15 region was present in the affected children. It has, therefore, been proposed that IC1 microdeletions are part of a cascade of molecular defects and, if isolated, may not necessarily lead to BWS (23).

To further investigate the molecular mechanism causing the imprinting defect of *IGF2* and *H19* and to define the role of the IC1 sequence elements in BWS pathogenesis, we looked for additional cases with mutations in IC1. In this article, we report two novel families with multiple individuals carrying 1.4 and 1.8 kb deletions of IC1. In addition, we analysed the methylation at the seven CTSs of the IC1 region and the *IGF2* and *H19* genes together with the clinical phenotypes in all the individuals with maternal transmission and the majority of those with paternal transmission of five different deletions. The results obtained demonstrate that IC1 deletion is a cause of familial BWS characterized by dominant maternal transmission and loss of *IGF2-H19* imprinting and high risk of developing Wilms' tumour. We also show that the penetrance of these mutations correlates with the hypermethylation gained by the mutant IC1 and that this may depend on the spacing of the remaining CTSs on the deleted allele. Finally, mosaicism for the methylation defect suggests that the epigenetic defect is acquired post-zygotically and its extent may influence the clinical phenotype.

RESULTS

Previous analyses showed the presence of microdeletions in the IC1 region of the 11p15.5 imprinted gene cluster in a few patients with BWS (12,14,24). The deletions eliminated from 1.4 to 2.2 kb of the IC1 region, including from one to three CTSs. In all cases, the BWS phenotype was observed

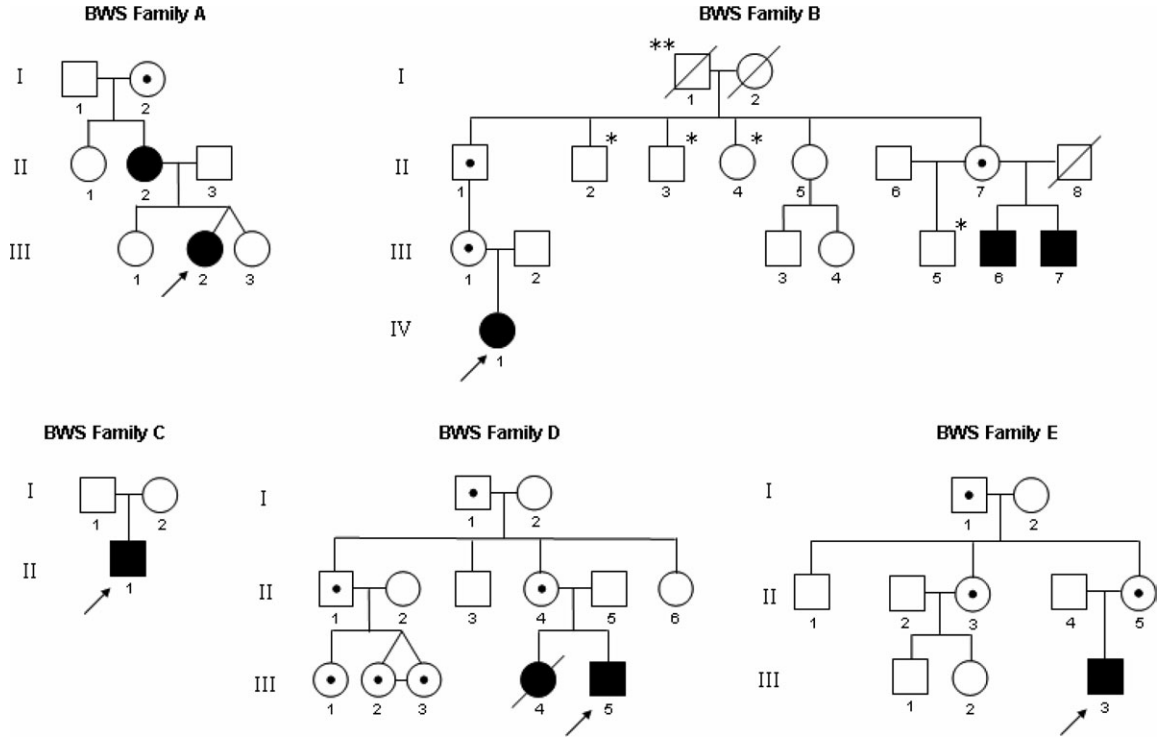

Figure 1. Pedigrees of five kindreds with IC1 microdeletions and familial BWS. Carriers of the IC1 deletions are indicated. *Individuals with normal phenotype were unavailable and could not be tested. **Paternal transmission of the deletion was inferred by IC1 methylation in II1 (see Results). Families BWS C–E were previously reported (12,24). Note that maternal transmission segregates with the BWS phenotype with complete penetrance.

only in the individuals with maternal transmission of the deletion. However, in the family described by Prawitt *et al*. (14), the mutation was not always associated with the disease, raising the hypothesis that the microdeletions could only predispose to BWS and additional molecular defects were necessary for overt clinical expression. More intriguingly, hypermethylation of the residual IC1 sequence was not consistently associated with the deletions (23,24). In order to determine the critical IC1 elements and the mechanism by which their mutation causes BWS, we sought to identify additional patients carrying microdeletions in this region.

Clinical evaluation of patients with IC1 microdeletions

After screening 100 patients with the clinical diagnosis of BWS, two novel families including five affected individuals carrying IC1 deletions were identified (Fig. 1, BWS families A and B). The pedigrees of the three families previously reported by our group are also shown in Figure 1 (BWS families C–E). Case reports are presented in Materials and Methods. Overall, nine patients with BWS and IC1 microdeletions are present in these families. In all cases, the mutation was maternally inherited and all the individuals with maternal inheritance showed the BWS phenotype. In II-1 of the BWS family C, the IC1 deletion was *de novo* and probably arose during maternal gametogenesis. The individual I-1 of the BWS family E showing normal phenotype was mosaic for the presence of the IC1 deletion on his paternal chromosome (data not shown). The clinical features of all the patients with IC1 microdeletions were compatible with the characteristics more often found in the BWS cases with defects of the IC1 domain (22). In particular, they all had macroglossia and macrosomia at birth, although abdominal wall defects were absent or not severe and the incidence of Wilms' tumours was high (2/6 at 6 years, with nephromegaly present in two additional cases of 2 and 3 years). Interestingly, hemihypertrophy (particularly of the face) was present in five of eight patients and an additional one had congenital stiff neck. No BWS feature or growth abnormality was evident in the 13 individuals with paternal inheritance of the IC1 deletions or in any of the family members that did not inherited the mutations, thus showing that dominant transmission with complete penetrance of the BWS phenotype occurs upon maternal inheritance of the IC1 microdeletions in these families.

IC1 microdeletions

Microdeletions were detected by long-range polymerase chain reaction (PCR) with primers flanking the repetitive region of IC1 and Southern blotting (12). Deletions breakpoints were identified by sequencing the deleted alleles. The human IC1 region includes two large repeated units, each composed of a 450 bp A-type repeat and several 400 bp B-type repeats (Fig. 2) (25). Six of seven IC1 element are present in the B-type repeats. Given the organization of IC1 by a tandemly repeated array of blocks with high sequence homology, mispairing of homologous chromosomes or sister chromatids

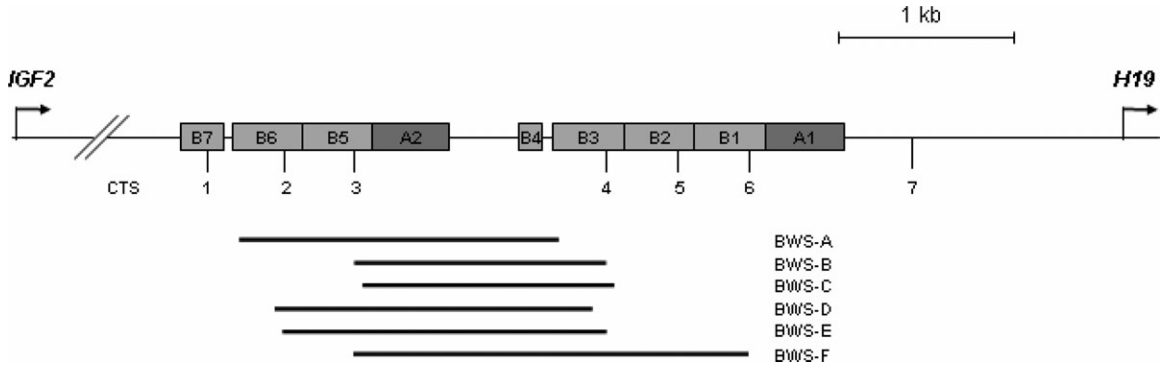

Figure 2. Summary map of the IC1 deletions. The extension of each deletion is shown by a bar with new cases in boldface (BWS-A and BWS-B). The A- and B-repeats of the human IC1 are shown as grey boxes. The positions of the CTCF target sites (CTSs) and the *IGF2* and *H19* transcription start sites are indicated. The deletions BWS-C/E were previously reported by our group (12,24). The larger BWS-F deletion that was associated with BWS with incomplete penetrance (14) is shown for comparison. Sizes and breakpoints of the IC1 deletions are reported in Table 1. Note that all the deletions disrupt the bipartite structure of the IC1 region, but only the BWS deletions F retain the three CTS-cluster organization characteristic of the wild-type allele.

Table 1. Different types of deletions in the human IC1 region

IC1 deletion	Extension (bp)	Breakpoint range[a]	Fused repeats
BWS-A	1834	5067–5086/6901–6920	B6/B3
BWS-B	1433	5710–5720/7143–7153	B5/B3
BWS-C	1433	5723–5752/7156–7185	B5/B3
BWS-D	1834	5275–5287/7109–7121	B6/B3
BWS-E	1834	5297–5314/7131–7148	B6/B3
BWS-F	2245	5710–5721/7955–7966	B5/B1

[a]GenBank accession no. AF125183.

may drive non-allelic homologous recombination (NAHR), i.e. unequal crossover/chromatid exchange during meiosis/mitosis leading to deleted and duplicated chromosomes. This type of exchange is reciprocal, but we evidence herein the pathogenic effect of repeat losses within IC1. The extensions of the deletions are shown in Figure 2, whereas their exact sizes and breakpoints are reported in Table 1. The deletion present in the BWS family A is 1.8 kb long and is generated by recombination of the B6 with the B3 repeat. This eliminates almost the entire B6 repeat together with the B5, B4 and A2 repeats. The BWS-B deletion is 1.4 kb long and is generated by recombination between the B5 and the B3 repeats. The A2, B4 and most of the B3 repeats are eliminated in this case. The deletions previously reported by our group [BWS-C, BWS-D and BWS-E (12,24)] and others [(BWS-F (14)] are also shown in Figure 3, for comparison. The BWS-B and BWS-C deletions are very similar in terms of width and location of breakpoints, whereas the BWS-A deletion has the same width of BWS-D and BWS-E, but its breakpoints are located 200 bp more 5′. The BWS-F deletion is larger (2.2 kb) than the other ones and is the only one eliminating three B-repeats and one A-repeat. No such deletion was found in about 200 unrelated control individuals. It has been described that the BWS-F mutation was accompanied by 11p15.5 duplication in the individuals with the BWS phenotype (14). We genotyped the patients carrying the deletions BWS-A/E by microsatellite and restriction fragment length polymorphism (RFLP) analysis (*TH* and *IGF2* VNTRs and *IGF2 Apa*I, *H19 Alu*I and *Rsa*I RFLPs) and found no evidence of 11p15.5 duplication in any of them (data not shown).

Some of the CTSs are lost with the deletions. In particular, the deletions BWS-A, BWS-D and BWS-E abolish CTSs 2 and 3, the deletion BWS-C eliminates only CTS 4, whereas CTSs 3, 4 and 5 are lost with the BWS-F deletion. The deletion BWS-B loses one CTS by fusing CTS 3 and CTS 4. Although the breakpoints of the six deletions are different, in all cases, the normal IC1 structure consisting of two large repeated units is disrupted (Fig. 2). Consistent with the previous observations (24), only the deletion BWS-F reported by Prawitt *et al*. (14) reconstitutes a cluster of three CTSs that is similar to the ones present in the wild-type allele, whereas all the other deletions (that abolish one or two CTSs) create longer CTS clusters.

DNA methylation analysis

The DNA methylation defects of the *H19* DMR and promoter are routinely detected by Southern blotting in BWS (22). However, the CpG dinucleotides analysed by this procedure do not correspond with the recognition sequences for the putative regulatory factors of the IC1 region (26). Previous analyses by bisulphite-sequencing procedures have provided contrasting results on the normal methylation status of some of the CTSs of IC1 (12,27). We have now analysed the DNA methylation of each of the seven CTSs and of the *H19* promoter in the leukocyte DNA derived from 40 control individuals by methylation restriction (MR)-PCR. This procedure consists of treatment of the genomic DNA with sodium bisulphite, PCR amplification and digestion with restriction enzymes discriminating the methylated from the non-methylated target sites. The results demonstrated $50 \pm 5\%$ methylation at all CTSs and *H19* promoter, consistent with the paternal-specific methylation of all these sequences in normal individuals (Fig. 3). We then used the same method to analyse the individuals carrying the IC1 deletions. Figure 3 shows the results obtained from the individuals with maternal and paternal transmissions of the microdeletions in the BWS families A–E. CTSs 2 and 3 are abolished by the deletions BWS-A, BWS-D and BWS-E, whereas CTS 4 is

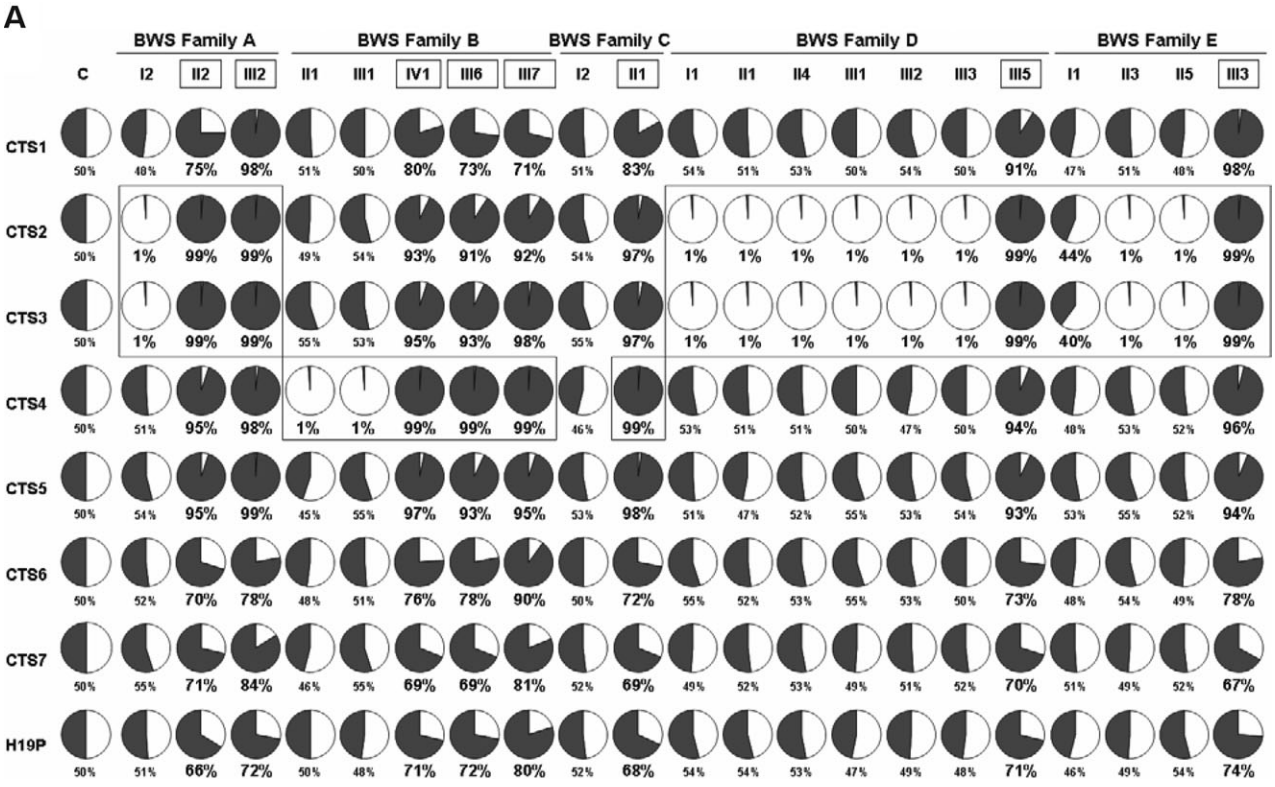

Figure 3. Methylation of the CTSs and *H19* promoter upon maternal and paternal transmissions of the IC1 microdeletions in the BWS-A–E families. (**A**) Summary of the results obtained on 40 control individuals, eight individuals with maternal transmission and 12 individuals with paternal transmission of the 1.4–1.8 kb deletions. DNA methylation at CpGs included in the CTSs and *H19* promoter was assayed by MR-PCR in leukocyte DNA derived from individuals with maternal (boxed) or paternal inheritance of the IC1 deletions. The average methylation levels detected in 40 control individuals (C) are shown for comparison. The extent of methylation at each CpG site is indicated with pie charts filled in black. The pies corresponding to CTSs eliminated by the IC1 deletions are framed. In family B, CTS 3 is a hybrid between CTS 3 and CTS 4 on the deleted allele. Methylation levels differing from controls by >1 SD (5%) are in boldface. (**B**) Examples of the assays whose results are summarized in (A). Lanes obtained with a control individual (C), an individual with paternal transmission of a 1.8 kb deletion (PatΔ1.8), an individual with maternal transmission of a 1.8 kb deletion (MatΔ1.8), an individual with maternal transmission of a 1.4 kb deletion (MatΔ1.8) and an individual with paternal transmission of a 1.4 kb deletion (PatΔ1.8) are shown for each CTS and for the *H19* promoter. A diagram with the restriction sites analysed (an asterisk indicating the one corresponding to the CTS) is shown above each panel. In each gel, the full-length fragments derive from non-methylated DNAs and shorter fragments from methylated DNAs. B, *Bst*UI; T, *Taq*I. Note that maternal transmission results in incomplete hypermethylation at all CTSs and *H19* promoter, whereas paternal transmission does not affect methylation.

eliminated by the deletions BWS-B and BWS-C. When only one parental allele is present, these CTSs appear completely unmethylated if maternal and completely methylated if paternal, confirming the imprinted methylation of these sequences (see CTSs 2 and 3 of II2 and III2 of family A, II1, II4, III1, III2, III3 and III5 of family D and II3, II5 and III3 of family E and CTS 4 of III1, III6, III7 and IV1 of family B in Figure 3). Therefore, the methylation patterns demonstrate that the deletions are paternally inherited in the individuals I2 of family A, II1 of family B and I1 of family D and maternally inherited in the individuals II1 of family C. Partial hypomethylation at CTSs 2 and 3 is present in I1 of family E because of mosaicism for the presence of the mutation on his paternal chromosome. Apart from these cases, the CTSs and the *H19* promoter showed $50 \pm 5\%$ methylation in the individuals inheriting the IC1 microdeletions from their father (Fig. 3). Similar results were observed in the individuals who did not inherit the deletions (data not shown). In contrast, the CTSs were methylated between 65 and 97% in the individuals with maternal transmission of the deletions (Fig. 3), indicating that hypermethylation was always associated with the deletions but incomplete. In addition, the extent of methylation of the seven CTSs and *H19* promoter was heterogeneous. As a general rule, the internal CTSs appeared to be more methylated than the external ones and often the CTS located most 5′ was more methylated than the two CTSs located most 3′ and *H19* promoter on the deleted alleles. These results demonstrate that the human IC1 is composed of seven paternally methylated CTSs and that maternal transmission of the deletions abolishing CTSs 2 and 3 or CTS 4 consistently leads to incomplete loss of the imprinted methylation at all the residual CTSs and *H19* promoter.

In the mouse, mutation of the maternal IC1 results in hypermethylation of the maternal *Igf2* DMR2 and *Igf2* activation (19,28). The human *IGF2* DMR2 is incompletely methylated on the paternal allele (29,30). We therefore wanted to assess whether the human IC1 deletions also led to the gain of methylation at *IGF2*. We analysed the methylation of two CpGs in this region by MR-PCR. The average methylation

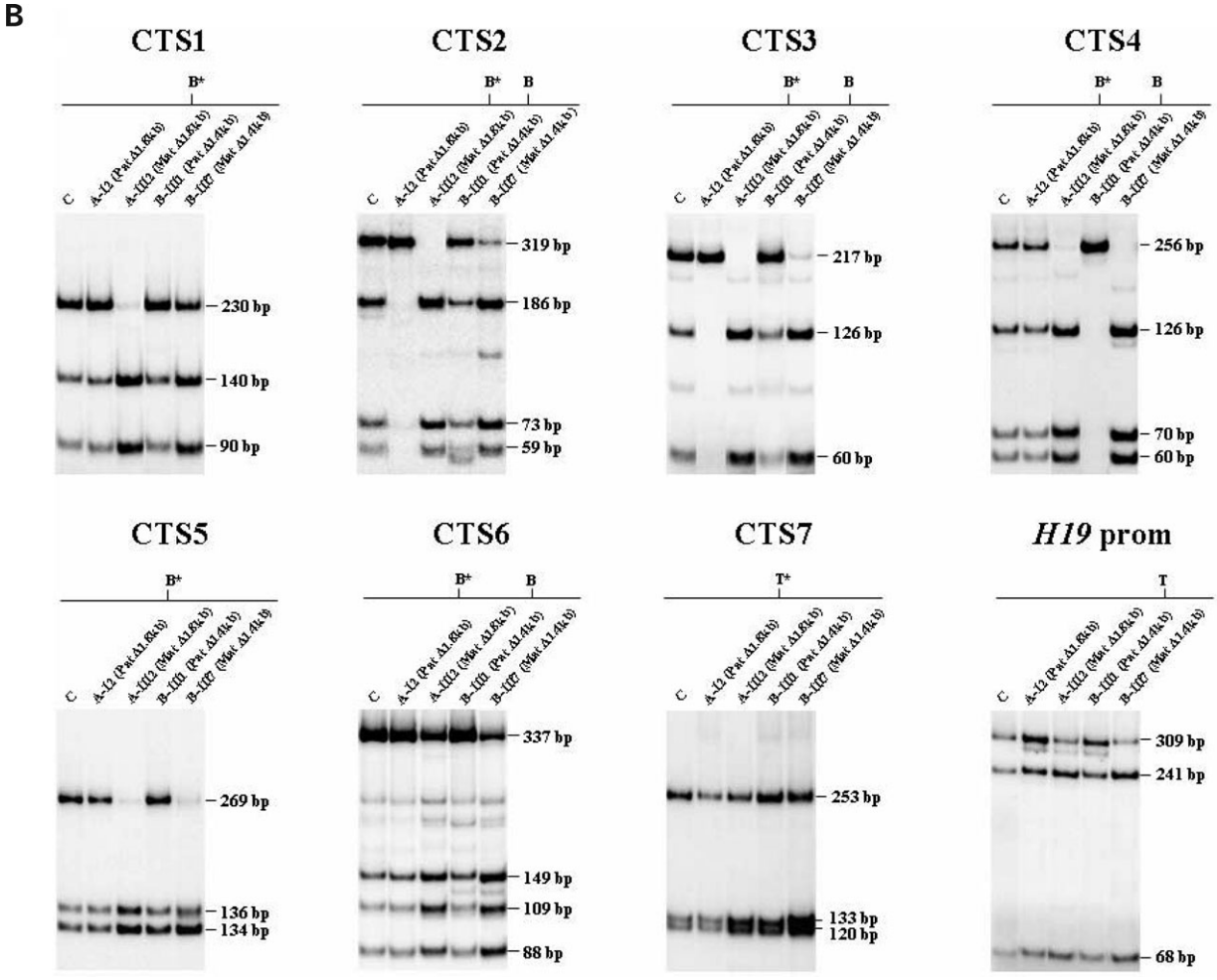

Figure 3. Continued.

was 39 ± 3% in 12 control individuals, although methylation ranged from 55 to 66% in all the individuals with maternal inheritance of the IC1 deletions (Fig. 4). In contrast, methylation levels similar to the controls were found in the individuals with paternal transmission and those not inheriting the deletions (data not shown). Overall, these data show that if maternally inherited, the 1.4–1.8 kb IC1 deletions consistently lead to disruption of imprinting at the *IGF2-H19* locus with the maternal allele adopting the epigenetic modifications that are characteristic of the paternal allele and normally associated with *IGF2* activation and *H19* silencing.

DISCUSSION

It was previously observed that BWS was associated with inherited IC1 microdeletions (12–14). However, it was debated whether these defects were sufficient or whether additional molecular lesions were needed to cause the disease phenotype (23,24). By analysing five families with nine affected individuals in multiple generations, we now demonstrate that maternal transmission of 1.4–1.8 kb deletions in the IC1 region cosegregates with the disease. In contrast, paternal transmission of these mutations is associated with normal phenotype. In addition, incomplete hypermethylation of the residual IC1 region, *H19* promoter and *IGF2* DMR2, is observed in all the individuals with maternal transmission, but none of those with paternal transmission of the mutations. These results support the hypothesis that IC1 microdeletions cause BWS with complete penetrance upon maternal transmission when they are associated with hypermethylation of the *IGF2-H19* locus.

In total, 10–15% of the cases with BWS are familial and heterogeneous modes of inheritance have been demonstrated (10). Many of the pedigrees reported show an autosomal dominant inheritance with preferential maternal transmission (31). Loss-of-function mutations in *CDKN1C* have been demonstrated in 40% of these cases (32,33). Inherited microdeletions in the ICs have been reported in some families with one generation of affected individuals (12–14). Our results now demonstrate segregation of IC1 microdeletions with the BWS phenotype within multiple generations, indicating IC mutation as an additional possible cause of familial BWS with autosomal dominant transmission. Because of the repetitive structure of the region (A- and B-repeats show 85.4 and 85–91% identity, respectively), NAHR between mispaired

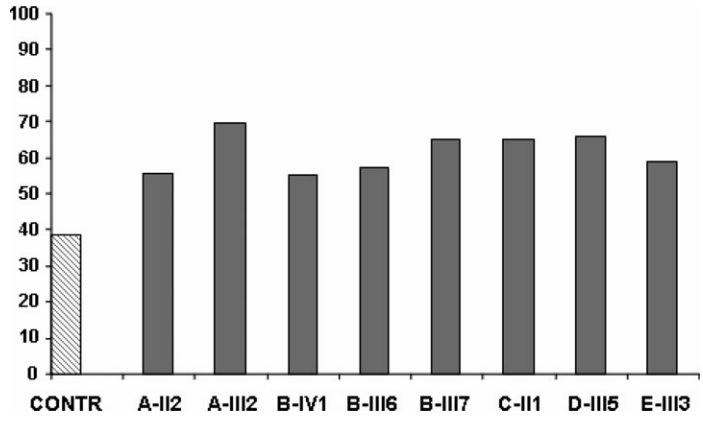

Figure 4. Effect of the IC1 microdeletions on methylation of the *IGF2* DMR2. DNA methylation at the *IGF2* DMR2 was assayed by MR-PCR in leukocyte DNA derived from the individuals with maternal inheritance of the IC1 deletions and controls (average of 12 individuals). The histogram shows the average methylation of two CpGs. The individuals with paternal inheritance of the IC1 deletions had methylation levels similar to the controls (data not shown). Note that maternal transmission of the IC1 microdeletions results in the gain of methylation at the *IGF2* DMR2.

blocks lying in direct orientation may generate recombinant deleted and duplicate alleles. IC1 deletions may be relatively frequent in some cohorts of patients, whereas pathogenic duplications have not been described so far. A decade of research on the mechanisms leading to genomic diseases has shown that the phenotypic effects associated with duplications are generally different from those consequent to deletion of the same paralogous structures and more subtle to disclose (34). Indeed, we found IC1 deletions at least as frequent as *CDKN1C* mutations among the Italian BWS patients analysed, suggesting that in a few cases, BWS is a genomic disease, resulting from errors primed by the architectural motifs of the IC1 genomic structure. The clinical phenotype of the patients with IC1 microdeletions is similar to the one described for the patients with sporadic hypermethylation at *IC1/H19* (22). The high incidence of Wilms' tumour makes the diagnosis of these mutations particularly relevant for genetic counselling.

It has been shown in the mouse that the CTSs within the IC1 element are required for its insulator function and confer protection against *de novo* methylation in somatic cells (26,35–37). Furthermore, CTCF binding controls the interaction between IC1 and the *Igf2* DMRs that are required to partition the *Igf2* and *H19* genes into transcriptionally active and inactive chromatin loops in a parent of origin-dependent manner (19,21). Mutation of the maternal CTSs leads to IC1 hypermethylation, loss of interaction with the *Igf2* DMR1 and gain of interaction with the *Igf2* DMR2 that also become hypermethylated as normally occurs on the active paternal *Igf2* allele (19,28). We observed hypermethylation of the IC1 CTSs and *IGF2* DMR2 in all BWS patients with IC1 microdeletions. Although there may be some differences between the epigenetic marks of the mouse and human *IGF2-H19* locus (30), it is likely that the epigenetic alterations resulting from the microdeletions lead the maternal 11p15.5 locus to acquire high-order chromatin structures that are typical of the paternal chromosome and associated with *IGF2* activation and *H19* silencing. Consistent with this conclusion, biallelic activation of *IGF2* and silencing of *H19* were demonstrated in tissue biopsies of the patients D-III5 and E-III3 (12).

Prawitt *et al*. (14) described a family with a 2.2 kb deletion of IC1 characterized by incomplete penetrance of the BWS phenotype upon maternal transmission. Indeed, one of the individuals with the disease was shown to have an additional mutation consisting in duplication of the 11p15.5 region. We found that maternal transmission of the 1.4–1.8 kb deletions is associated with complete penetrance of the clinical phenotype and none of the nine affected individuals has evidence of 11p15.5 duplication. We previously observed that a possibly important feature associated with the 1.4–1.8 kb deletions, but not with the 2.2 kb deletions, was the hypermethylation of the residual IC1 sequence and proposed that this was caused by the loss of the normal spacing of the CTSs on the alleles with the shorter deletions (24). Although we cannot exclude the presence of additional point mutations at linked loci in our patients, the analysis of five additional cases and two novel deletions is consistent with the hypothesis that IC1 hypermethylation is the causative defect. Indeed, the abnormal methylation is always associated with maternal transmission of the 1.4–1.8 kb deletions. In addition, these mutations always disrupt the structure of the normal IC1 element by creating abnormally long (more than three sites) clusters of CTSs. In contrast, the normal three CTS-cluster organization, with the loss of one large repetitive unit, is maintained by the 2.2 kb deletion allele described by Prawitt *et al*. (14). Although the involvement of factors in addition to CTCF cannot be excluded, the more intense hypermethylation of the internal CTSs suggests that these have lower affinity for CTCF on the mutant IC1 alleles and the long (more than three) CTS clusters have a spacing problem resulting in the gain of methylation. It is likely that IC1 hypermethylation leads to complete inactivation of the insulator function, *IGF2* activation and *H19* silencing and this probably explains the complete penetrance of the 1.4–1.8 kb deletions. Conversely, the non-methylated 2.2 kb deletion allele probably retains residual insulating activity and is associated with modest *IGF2* activation and normal *H19* expression (38). Therefore,

the latter deletion might require additional mutations to significantly alter *IGF2* transcription and cause BWS.

Our study clearly shows that all of the IC1 CTSs are differentially methylated in the normal human population, as it has been previously demonstrated in the mouse. This suggests that each site (or most of them) binds CTCF on its non-methylated maternal allele and contributes to the IC1 function. After this work was completed, Tost *et al*. (39) reported a rather surprising result on the analysis of IC1 methylation in the normal French population. By employing different methods, they demonstrated lack of differential methylation at the sixth CTS (either hypo- or hypermethylation) in DNAs derived from different tissues of ~40% of the individuals tested. They also found that the epigenetic profile was genetically determined. However, the abnormal methylation did not extend to the other CTSs and *H19* promoter and was associated with maintenance of *IGF2* and *H19* imprinting. A previous study also reported abnormal methylation of the sixth CTS (although at a lower frequency) in non-pathological conditions and with familial aggregation (40). We have not found any deviation from the expected 50% methylation in the 40 healthy subjects used as controls and 12 individuals with paternal transmission of the microdeletions whose IC1 methylation was analysed by MR-PCR. In addition, the differential methylation of the sixth CTS was confirmed by bisulphite sequencing and allele discrimination in 10 normal subjects (data not shown). Although the polymorphic nature of the methylation of a single CTS in the normal population remains a possibility, these results support the hypothesis that IC1 function derives from the activities of multiple sites and can probably tolerate the inactivation of a single CTS. Problems in clinical analysis can easily be avoided by assaying methylation at more than one CTS.

In principle, imprinting defects at ICs can derive from failure of erasure, establishment or maintenance of the imprint (1). IC microdeletions causing lack of establishment of the maternal methylation have been described in Angelman syndrome, whereas IC mutations resulting in inefficient maintenance of the paternal non-methylated status have been found in Prader–Willi syndrome (41). The incomplete hypermethylation of the CTSs found in the BWS patients with 1–4–1.8 kb deletions indicates mosaicism for the imprinting defect and suggests that the methylation is acquired post-zygotically and results from insufficient protection from *de novo* methylation of the maternal IC1. Consistent with this hypothesis, targeted mutations of the IC1 CTSs in the mouse demonstrate that these sequences are dispensable for imprint establishment in the gametes, but are needed for proper imprint maintenance in somatic cells during embryo development (26,36).

If inappropriate imprint maintenance in early embryogenesis results in mosaicism for the imprinting defect, the phenotype of the individuals carrying the IC1 deletions may vary according to the degree of mosaicism and the type of tissues more intensely affected by the epigenetic defect. We did not observe a clear relationship between the extent of hypermethylation at the *IGF2-H19* locus and the severity of the BWS phenotype. However, it is possible that the degree of methylation of leukocyte DNA is insufficient to predict the extent of mosaicism in the whole body and tissues, more important for the BWS phenotype should be examined. The high incidence of hemihypertrophy suggests indeed that at least some of the clinical features of the patients with IC1 deletion are influenced by mosaicism. Body asymmetry in BWS was previously associated with mosaicism for paternal 11p15.5 UPD (22). Our results now indicate that hemihypertrophy can also be caused by epigenetic mosaicism at an IC.

Deletion of the paternal IC1 leads to *Igf2* down-regulation and growth retardation in the mouse (42–44). It is unclear if this is due to a direct positive function of the methylated paternal IC1 (possibly mediated by interaction with the *Igf2* DMR2) or uncontrolled competition with the *Igf2* and *H19* promoters for shared enhancers. In addition, it has been recently shown that growth restriction phenotypes are associated with hypomethylation of the 11p15.5 IC1 (45,46). This prompted us to check whether the human IC1 deletions cause any growth restriction in humans upon paternal transmission. However, we observed normal growth phenotype in the 13 individuals inheriting the IC1 deletions from their father. Likewise, no methylation abnormality of the *Igf2-H19* locus was found, indicating that the function normally exerted by IC1 on the paternal chromosome is not altered by the BWS microdeletions.

In summary, our studies demonstrate a novel mechanism for an autosomal dominant form of familial BWS with high incidence of Wilms' tumour, in which inherited microdeletions alter the imprinting maintenance of the *IGF2-H19* locus at post-zygotic stages. Pre-natal genetic testing should be considered for such families, as the recurrence risk may be as high as 50% upon maternal transmission. Defective imprinting of *IGF2-H19* has been described in several BWS cases and cancers with no associated IC1 deletion and lower recurrence risk (6,47). The mechanism causing the loss of imprinting in these cases remains to be demonstrated.

MATERIALS AND METHODS

Clinical reports and genealogical analysis

Brief reports for patients with novel molecular findings are presented here to allow phenotype–genotype correlations. Figure 1 depicts the pedigrees of the investigated BWS patients (families A–E).

BWS family A **Case III-2**. The propositus was born to unrelated parents from a dizygotic twin delivery. The twin sister and an older sister are normal. Birth weight was 3100 g, whereas her twin weighed only 2100 g. The child was transferred to a cardiology unit because of respiratory distress because of ventricular septal defect (VSD) with patent ductus arteriosus. When the patient was admitted to the neonatal intensive unit, the diagnosis of BWS was placed on the basis of the presence of macrosomia with relative microcephaly (weight and length 97th centile and OFC 25th centile), macroglossia, capillary haemangioma on the glabella, chubby cheeks, prominent eyes, slight asymmetry of the face and thorax, ear creases and hypoglycaemia. The VSD closed after the pharmacological therapy and the hypoglycaemia was corrected with frequent feeding. Since birth, she has undergone follow-up with periodical abdominal ultrasound monitoring and alpha-fetoprotein (AFP) level assay in order

to detect early tumour onset. At 1 year, a first-stage Wilms' tumour was diagnosed and surgically removed. Psychomotor development was slightly delayed when compared with her twin sister. At 6 years, the clinical phenotype was unchanged.

Case II-2. At birth, the mother of III-2 was admitted to the hospital because of suspected Down syndrome. She had macrosomia (birth weight was 5000 g and that of her older sister was 3100 g) and protruding tongue. She underwent surgical correction of congenital stiff neck. At 30 years, her height was at the 90th centile. She had chubby cheeks and prominent eyes but no macroglossia, ear creases or pits and body asymmetry. She showed some behaviour disturbances and her IQ was slightly reduced. Her sister (II-1) and her mother (I-2) had normal phenotype.

BWS family B **Case IV-1**. The female patient IV-1 was conceived by *in vitro* fertilization to unrelated parents. Polyhydramnios was reported during pregnancy. She was born by caesarean section at the 33rd week of gestation. At birth (APGAR index 3 at first minute, 9 at 5th minute), she was subjected to cardiopulmonary resuscitation for respiratory distress. BWS diagnosis was placed on the basis of the presence of macrosomia (birth weight 3710 g, birth length 52 cm and OFC 31 cm), macroglossia and slight left facial hemihypertrophy. Mild dysmorphisms such as slight ephicantus were present. No haemangioma or abdominal anomalies were observed. Genetic counselling revealed two mother's first cousins with BWS (III-6 and III-7). Since birth, she has undergone follow-up with periodical ultrasound monitoring. Until last examination (6 years), she kept on growing over 97th centile (30.1 kg and 135 cm) with no physical anomaly being reported, apart from a dysplasic form of left kidney. Macroglossia and face asymmetry were still present, whereas no asymmetry in the lower limbs could be noticed. Psychomotor development was proper to her chronological age. The phenotypes of her mother (III-1) and maternal grandfather (II-1) were normal.

Cases III-6 and III-7. The maternal uncles of case IV-1 are brothers born to unrelated parents, III-6 by caesarean section at 40 weeks of gestation. Pregnancy went on without peculiar problems in both cases. Overgrowth and macroglossia were reported at birth. Normal psychomotor development was observed, whereas follow-up and ultrasound monitoring did not reveal any anomaly. Both patients showed similar facial dysmorphisms including brachycephalia, oval face with hemihypertrophy (left in III-6 and right in III-7), prominent arched and beetle eyebrow, synophrys, deeply set eyes, prominent nose, slight malar hypoplasia, pronounced filter, cleft palate and dental anomalies. No limb hemihypertrophy could be noticed. Posterior helical pits, umbilical hernia and diastasis recti, slight cardiomegaly and splenomegaly were reported in III-6, whereas III-7 only showed mild diastasis recti, slight hepatomegaly and criptorchidism. The phenotype of their mother (II-7) was normal.

BWS family C **Case II-1**. This case was already reported by Cerrato *et al*. (24). Family history is unremarkable. The propositus was born by caesarean section at 38 weeks of gestation complicated by fetal overgrowth. Birth weight was 4490 g, length 56 cm and OFC 34 cm. There were no perinatal complications. BWS was suspected because of the presence of macrosomia and macroglossia. He underwent tongue reduction at 12 months of age. His psychomotor development was within normal limits. Abdominal echography showed enlarged kidneys. Trimestral screening with alpha-fetoprotein, urinary catecholamines and NSE showed no anomaly. At 22 months, his weight was 17 kg, height 99 cm (both values above the 97th centile) and OFC 48 cm (50th centile). He had residual macroglossia and mandibular prognathism, but no evidence of hemihypertrophy.

BWS family D **Case III-5**. This case was already reported by Sparago *et al*. (12). Both parents are healthy and consanguinity is denied. The propositus was born at 36 weeks of gestation complicated by fetal overgrowth. Birth weight was 4300 g, length 52 cm and OFC not recorded. The diagnosis of BWS was placed on the basis of the presence of macrosomia, macroglossia, neonatal hypoglycaemia and left megaureter. The neonatal hypoglycaemia was treated with i.v. glucose. The macroglossia and megaureter were surgically treated at 2 years. Psychomotor development was within normal limits. The child developed gross haematuria at 4.5 years and renal sonogram revealed a mass suggestive of Wilms' tumour in the left kidney. He underwent left nephrectomy and 6 months of chemotherapy. Follow-up with monthly renal ultrasound, alpha-fetoprotein and urinary catecholamines screening was negative. At 2 years, the child had macrosomia (weight 20 kg, length 99.5 cm and OFC 51 cm; all parameters well above 97th centile), hypertelorism, residual macroglossia with prognathism and diastasis recti. At 6 years, his weight was 40 kg, height 128 cm and OFC 55 cm (all values much above the 97th centile).

Case III-4. The sister of III-5 was born at 6 months of gestation with a birth weight of 2660 g. She had enlarged abdomen and macroglossia and died at 1 h because of respiratory distress. Her mother (II-4) and the other family members showed normal growth phenotype with no sign of BWS.

BWS family E **Case III-3**. This case was already reported by Sparago *et al*. (12). Both parents are healthy and consanguinity is denied. The propositus was born after 37 weeks of gestation complicated by polyhydramnios. Nephromegaly was detected at the 30th week of gestation by ultrasound echography. Delivery was accomplished by caesarean section. Birth weight was 3640 g, length 50.9 cm, OFC 31.3 and APGAR scores 6/8. He had neonatal hypoglycaemia and jaundice, macroglossia and a small umbilical hernia.

At 3 months, the clinical diagnosis of BWS was made on the basis of macrosomia, macroglossia and minimal hemihypertrophy ($R>L$ by 1 cm). His developmental milestones were delayed: he walked at 21 months and spoke at 3 years. MRI of the brain and EEG were normal. At 3.5 years, his weight was 23 kg (well above the 97th centile), length 106 cm (greater than 97th centile) and OFC 50.5 cm (50th centile). He had macroglossia, prognathism and slight hemihypertrophy. His mother (II-5) and the other family members showed normal growth phenotype with no sign of BWS.

All the genetic analyses were performed after the informed consent was obtained from the parents of the patients.

Table 2. MR-PCR conditions for the methylation analysis of the IC1 region

PCR primers	Annealing temperature (°C)	$MgCl_2$ (mM)	Digestion (no. of restriction sites)
CTS1_F 5′-GTATTTTTGGAGGTTTTTTATTTAG-3′ CTS1_R 5′-ACACCTAACCTAAAAAACCTAAAAC-3′	55	1.5	*Bst*UI (1)
CTS2_F 5′-AGGTGTTTTAGTTTTTTGGATGATA-3′ CTS2_R 5′-CCATAAATATTCTATCCCTCACTA-3′	60	1.5	*Bst*UI (2)
CTS3_F 5′-GGTTTTTGGTAGGTATAGAAATTG-3′ CTS3_R 5′-CACCTAACTTAAATAACCCAAAAC-3′ CTS3_R(BWS-B, C) 5′-AACCCAAAACRTTTCCRCRAACRAACC-3′	62	1.5	*Bst*UI (2)
CTS4_F 5′-GTTTTTGGTAGGTTTAAGAG-3′ CTS4_F (BWS-D, E) 5′-TTTATAGGGTTTTTGGTAGG-3′ CTS4_R 5′-TAAATATCCTATCCCTAATAAC-3′	58	1.5	*Bst*UI (2)
CTS5_F 5′-TTTTGTAGGGTTTTTGGTAG-3′ CTS5_R 5′-TCCCATAAATATCCTATACCTC-3′	59	1.0	*Bst*UI (1)
CTS6_F 5′-GAGTTTGGGGGTTTTTGTATAGTAT-3′ CTS6_R 5′-CTTAAATCCCAAACCATAACACTA-3′	58	1.5	*Bst*UI (2)
CTS7_F 5′-5′-GAGTATTTGTATTTTTGGAGTAT-3′ CTS7_R 5′-AAAAATTCTCAAACTTTTCCATAAA-3′	59	2.5	*Taq*I (1)
H19promoter_F 5′-TGAGGGAGGTGATGGGGTAATG-3′ H19promoter_R 5′-TTCCCCACTTCCCCAATTTCCC-3′	64	2.0	*Taq*I (1)

The experimental plan was approved by the Ethics Committee of the Second University of Naples.

Southern blot, PCR, DNA sequencing and microsatellite analyses

Southern blot hybridization was performed on DNA purified from blood leukocytes as described (12). PCR amplification of the *H19* DMR was obtained from leukocyte DNA by using the primers 5′-AGAGATGGGATTTCGTCAGGTT GG-3′ and 5′-CATTTCCGTCTCCACAGCCACAAC-3′ and the Taq BIO-X-ACT Long (BIOLINE) as described (12). Thefragments generated from the allele carrying the microdeletions were gel-purified and cloned onto pCR II (Invitrogen). DNA sequencing was obtained from PRIMM (Italy). Long PCR product of family B's individuals was directly sequenced on ABI Automated Sequencer (mod 3100) by using primers forward: 5′-GGATGATGGGGATCT C-3′ and reverse: 5′-GGATCTCACCCTGTGGCCAA-3′ (AF125183) after restriction digestion with *Mbo*I. Microsatellite analysis was performed as previously described (48,49).

DNA methylation analyses

DNA methylation of the *H19* DMR and promoter was analysed by *Hpa*II digestion and Southern blot hybridization, bisulphite sequencing and bisulphite treatment coupled with restriction enzyme digestion (MR-PCR or COBRA). Sodium bisulphite treatment of DNA was performed as described (12). The PCR primers, conditions and restriction enzymes used are reported in Table 2. The methylation of the human *IGF2* DMR2 region was analysed by MR-PCR. After DNA modification with sodium bisulphite, the PCR amplification was carried out with the following primer pair: *IGF2* DMR2 F 5′-GGAAGAGYGTGGAGAGTAGGTATTTGTTG-3′ and *IGF2* DMR2 R 5′-ACTCACTTCCRATTACTAACCATCT C-3′. The annealing temperature was 59°C with 1.5 mM of $MgCl_2$. The PCR product was digested with *Taq*I or *Acl*I restriction enzymes and fragments separated on a non-denaturing polyacrylamide gel.

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Conflict of Interest statement. None declared.

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