Imprinting mutation in the Beckwith–Wiedemann syndrome leads to biallelic IGF2 expression through an H19-independent pathway

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The Beckwith–Wiedemann syndrome (BWS) is genetically linked to chromosome 11p15.5, and a variety of observations suggest that deregulation of imprinted genes in this region is causally involved in the pathogenesis of the disease. It has been shown that in some patients without cytogenetic abnormalities the otherwise repressed maternal copy of the insulin-like growth factor 2 (IGF2) gene is expressed, leading to biallelic expression of IGF2. In some of these cases, this is accompanied by repression and DNA methylation of the maternal (otherwise active) copy of the neighbouring H19 gene. Hence, it is attractive to think that mutations may interfere with some aspect of H19 imprinting, thus leading to an inactive maternal allele, and indirectly to activation of the maternal IGF2 allele as reported in mice with an H19 gene deletion. However, no mutations have been identified so far in these patients. The only known mutations associated with BWS are maternally transmitted translocations, which are clustered in two locations centromeric to IGF2. The first cluster is 200–400 kb from IGF2 and the second is several megabases away. Hence, genes located far from the translocation breakpoints are potentially deregulated by them. Here we provide the first evidence of alteration of imprinting in a translocation family, with biallelic expression of IGF2 and altered DNA replication patterns in the IGF2 region. Interestingly, H19 imprinting was normal, suggesting an H19-independent pathway to biallelic IGF2 transcription. DNA methylation in IGF2 remained monoallelic, suggesting that the mutation in this family had uncoupled allele-specific methylation from expression.

INTRODUCTION

The genetic regulation of genomic imprinting is of considerable importance for normal development and for genetic disease. Close physical clustering appears to be an important organisational feature of imprinted genes in mammals. In mouse and human there are at least two major imprinted gene clusters. One is located on human chromosome 11p15.5 and contains the imprinted genes IGF2, H19, P57kip2 and possibly others (1,2). The syntenic region in the mouse is on distal chromosome 7 and contains the same imprinted genes (1,2). The second region is on human chromosome 15q11–13 and on the middle part of mouse chromosome 7, and contains the imprinted ZNF127, DN34, PAR1, PAR5, IPW and SNRPN genes (3). There are also two closely linked imprinted genes on mouse chromosome proximal 17, Igf2r and mas (1,2). Other imprinted genes are in other regions and it is too early to say whether they are also in imprinting clusters.

The control of imprinting in these clusters is likely to require both regional cis-acting elements (imprinting centres, IC) as well as local cis-acting elements in individual genes. Indeed, some cis-acting and closely linked sequences have been shown to be important for aspects of the imprinting of the mouse H19 gene (4). Regional control through postulated ICs has been demonstrated in the Prader–Willi/Angelman syndromes (PWS/AS) where small deletions just 5′ of the SNRPN gene can affect allele-specific methylation and expression in a large domain on chromosome 15q11–13 (5,6). These imprinting mutations are thought to interfere with the setting or resetting of the imprinting signal in the germline (6). A recent interesting finding is that the homologous chromosomes in imprinted regions seem to physi
cally pair during mitosis, and imprinting mutations in PWS/AS may interfere with this pairing (7).

Regional control has also been shown for the Igf2-H19 domain in the mouse. Deletion of the active maternal H19 gene results in derepression of the maternal copy of Igf2, and deletion of one of the H19 enhancers on the paternal chromosome leads to decreased Igf2 expression on that chromosome (8,9). This might occur by the two genes sharing enhancers to which only one gene at a time has access.

There are a number of disease situations in the human that suggest that similar co-regulation of imprinting in this domain exists. Both in Wilms’ tumour of the kidney (10,11) and in the fetal overgrowth and childhood tumour syndrome, Beckwith–Wiedemann syndrome (BWS, 12), as well as in other overgrowth situations (13), hypermethylation of the maternal copy of H19 has been found in association with biallelic expression of IGF2. In none of these cases, however, have specific mutations been identified that lead to these altered patterns of imprinting. In addition, there are other developmental and pathological situations in which there is biallelic expression of one gene and monoallelic expression of the other, suggesting additional pathways of imprinting control (14–20). Again, in none of these situations have mutations been identified.

BWS provides a good model for the investigation of regional control in the IGF2-H19 imprinting domain. First, biallelic expression of Igf2 occurs in sporadic cases of the disease (12,21,22). Second, in some of these patients we have previously detected methylation and transcriptional repression of the maternal (otherwise active) copy of H19 (12). Third, there are known mutations in BWS families in the form of translocations. In patients these are always inherited on the maternal chromosome and they are clustered in two locations centromeric to IGF2 (23–26).

Here we undertake the first imprinting analysis in a translocation family. We show that expression of IGF2 is biallelic and that this is associated with an altered pattern of DNA replication in Igf2. Imprinting in H19, by contrast, remained unaffected. This is, therefore, the first definitive demonstration of an imprinting mutation in the IGF2-H19 domain, and it suggests that there are H19 independent pathways that are involved in Igf2 repression.

RESULTS

The BWS inversion family D

The BWS family D (Fig. 1) has an inversion in the more distal (closer to IGF2) breakpoint cluster, inv(11) (p11.2; p15.5) (26,27). Cytogenetic analysis has been documented previously for the mother, the grandmother and the affected daughter III 1 (27), and was carried out for all the other family members except the grandfather in this study. The carrier mother (generation II) is not affected, and methylation analysis of IGF2 showed that the inversion is on the grandpaternal chromosome (not shown) but no cytogenetic or molecular information was available for the grandfather. The two children who inherited the inversion from the mother are affected, the third child who did not inherit the inversion is not affected. Marker analysis was carried out for IGF2/Apal, IGF2/CA repeat and H19/RsaI as well as for other markers in the region (Fig. 1). In the first affected daughter, there was an apparent recombination event between the inversion and the IGF2-H19 region.

The inversion leads to altered imprinting of Igf2 but not H19

Amniocyte and fibroblast cell lines were derived from the BWS baby III 3, and IGF2 and H19 allele-specific expression was determined by RT–PCR and using the Apal and RsaI polymorphisms, respectively. The results show (Fig. 2A and B) that Igf2 was expressed from both parental alleles, while H19 expression was limited to the maternal allele and thus showed the normal imprinting pattern. Biallelic Igf2 expression was also confirmed using the IGF2 CA repeat polymorphism (not shown). Controls showed monoallelic expression of IGF2 (Fig. 2A).

Allelic methylation

Allelic methylation was assessed in both genes in various members of family D. For H19, allelic methylation was assessed by digestion of DNA with PstI and SmaI, which assesses the methylation in one SmaI site in the H19 promoter, and two SmaI sites in the 3′ region of the gene (28). The SmaI site in the promoter is allele-specifically methylated on the paternal chromosome in all tissues tested so far (28). The analysis shows that H19 methylation in affected members of the family is identical to that in non-affected members and controls, both in leukocyte DNA (all family members with the exception of the grandfather were tested) and in DNA from fibroblast cell lines (Fig. 2C and not shown). This analysis, together with the normal maternal expression of H19, suggests that H19 imprinting is entirely normal and is not affected by the inversion.

For the Igf2 gene, allelic methylation was assessed in the 9th exon of the gene, where normally only the paternal allele is methylated, both in the human and in the mouse (28,29). In this region, methylation can be visualised on both alleles separately through the use of the Apal/AvaII polymorphism (Fig. 2D). Igf2 methylation in BWS child III 3 fibroblast DNA was indeed on the paternal allele, but surprisingly was reversed to maternal methylation in placenta and amniocytes (Fig. 2D). Monoallelic
methylation of IGF2 was also observed in leukocyte DNA of all other family members (the grandfather could not be tested). Hence, allelic methylation in IGF2 was monoallelic in affected and non-affected family members, but somatic allele switching was observed in one affected child. Somatic allele switching has been observed previously for IGF2 and H19, both in normal individuals and in tumours (14,15,20).

Replication timing

The 11p15.5 region has been shown previously to replicate asynchronously, with an overall tendency for the paternal allele to be replicated and resolved into doublets earlier than the maternal allele (30,31). We found that uniparental paternal disomy (UPD) cases, but not putative imprinting mutations with methylated H19 and IGF2 genes, had a substantial shift towards earlier replication (12). To address the question of whether the maternally inherited inversion resulted in an altered pattern of DNA replication, replication timing was examined in lymphoblastoid cell lines from the carrier mother and from BWS child III 1 (Fig. 1), using fluorescence in situ hybridisation with a cosmid probe that spans the TH, INS and IGF2 gene. The results show (Fig. 3) that in the affected child with the maternally inherited translocation, replication timing was substantially shifted to an earlier pattern as compared with controls [BWS III 1: singlet/singlet (SS) = 24%, singlet/doublet (SD) = 31%, doublet/doublet (DD) = 44%; means of four control cell lines: SS = 40 ± 2%, SD = 32 ± 5%, DD = 28 ± 2%]. The replication pattern of a uniparental paternal disomy patient and of a putative imprinting mutation patient are also shown for comparison (Fig. 3). The replication timing in BWS III 1 was similarly advanced as in the UPD case. This is more advanced than predicted from two paternal copies. The mother of the BWS children in family D (Fig. 1), who carries the inversion on the grandpaternal chromosome, had a slight reduction of SS patterns and a corresponding increase in DD patterns (SS = 35%, SD = 30%, DD = 35%), but this was not statistically significant.

DISCUSSION

The most significant finding in this study is that of an altered imprinting pattern in the IGF2 domain in a BWS family with a maternally inherited inversion at ~300 kb centromeric to IGF2, inv(11) (p11.2; p15.5). By definition, this is an imprinting mutation and it is the first imprinting mutation discovered in BWS. The only other syndrome in which imprinting mutations have been described recently is PWS/AS.

Maternal inheritance of this BWS imprinting mutation apparently has two effects. First, the maternal allele of IGF2 is derepressed. Second, replication timing in the region is considerably advanced.

One of the mechanisms that can lead to biallelic IGF2 expression is deletion or inactivation of the maternal copy of H19 (8,10–13), and this is accompanied by biallelic methylation of the DMR2 region of IGF2 (the conserved region of paternal allelic methylation in the last two exons of the gene) (12). We were surprised, therefore, to see that H19 imprinting was entirely normal both at the level of allelic expression and allelic methylation, and was unaffected by the maternally inherited inversion. Clearly, this indicates that the mechanism of biallelic IGF2 expression in this family is independent of H19.

There are three principal possibilities in our view of how this mechanism might work. First, it is possible that a repressive
of the gene. It has been proposed previously that the differentially methylated regions 1 and 2 in IGF2 are silencers whose function could be modulated by DNA methylation (29,32–34). There is now some evidence from transgenic studies to support this concept (C. Graham, personal communication). Whether there is a remote silencer sequence as far away as the inversion breakpoint is unknown at present.

The third possibility is that the breakpoint interrupts a (maternally expressed) gene whose product is essential for silencing of the maternal IGF2 allele in addition to having an intact and expressed maternal H19 allele. This product could be, for example, a repressor that binds to the maternal copy of IGF2.

This last model receives some support from our observation that allelic methylation in IGF2 remained monoallelic. Thus, allele-specific methylation and allele-specific expression of IGF2 were apparently dissociated by the mutation. One of the simplest scenarios to explain this might indeed be a mutation in a repressor molecule, which would normally bind to the unmethylated maternal allele of IGF2 but would be prevented from binding to the paternal allele by DNA methylation. This could mirror the situation in the human IGF2R gene, which has allele-specific methylation but is only rarely monoallelically expressed (35).

Whatever the precise molecular explanation for our findings, they clearly reveal an imprinted genetic locus (which is not H19) that has a suppressor function on IGF2.

So far we have detected biallelic IGF2 expression in a majority of all sporadic (non-UPD) BWS patients but methylation of H19 in a minority (5–10%) of these (Table 1, ref. 36, see also ref. 37). Preliminary results also indicate that IGF2 methylation is monoallelic in those patients with normal H19 imprinting (unpublished). These results, therefore, also indicate that the mechanism of biallelic IGF2 activation is heterogeneous and can either proceed through an H19-dependent or -independent pathway. Further translocation families need to be analysed to elucidate these mechanisms in more detail.

However, our findings further strengthen the relationship between normal H19 methylation and expression and normal IGF2 methylation on the one hand, and maternal H19 methylation or deletion and increased IGF2 methylation on the other hand. The allelic methylation of IGF2, therefore, does not appear to be a simple consequence of IGF2 transcription, but instead seems to be regulated by the H19 locus (34).

Table 1. Altered imprinting of IGF2 and H19 in BWS patients

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Normal karyotype</th>
<th>Normal karyotype</th>
<th>Maternally inherited rearrangement</th>
<th>Paternal UPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2 expression</td>
<td>biallelic</td>
<td>biallelic</td>
<td>biallelic</td>
<td>2 × monoallelic</td>
</tr>
<tr>
<td>H19 expression</td>
<td>?</td>
<td>none</td>
<td>normal</td>
<td>none (2 × monoallelic)</td>
</tr>
<tr>
<td>IGF2 methylation</td>
<td>normal</td>
<td>altered</td>
<td>normal</td>
<td>2 × monoallelic</td>
</tr>
<tr>
<td>H19 methylation</td>
<td>normal</td>
<td>altered</td>
<td>normal</td>
<td>2 × monoallelic</td>
</tr>
<tr>
<td>Replication</td>
<td>?</td>
<td>normal</td>
<td>altered</td>
<td>altered</td>
</tr>
<tr>
<td>Frequency</td>
<td>70%</td>
<td>5–10%</td>
<td>1%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Summarised are the findings from this and other studies (12,21,22,28,36,42). The first column shows the majority of BWS patients in whom no cytogenetic abnormalities are detected. In the majority of these, biallelic IGF2 expression and normal H19 imprinting is observed (E.R.M. et al., unpublished, ref. 37). The asterisk indicates that the majority of patients show biallelic IGF2 expression. It is important to recognise that there may be potential overlap in this category with other overgrowth syndromes such as Simpson–Golabi–Behmel (43).

The second column shows the class of patients in which there is biallelic IGF2 expression and biallelic inactivation of H19, without alteration of DNA replication (12). The third class of patients are the translocation patients reported here. The fourth class are uniparental paternal disomies.
lends further support to our suggestion that the two phenomena other mutations upstream of the described here with those found in PWS/AS. Small deletions and in the germlines to reset the grandparental epigenotypes (5,6). It has been proposed that this IC is required in being present on a paternal chromosome (in PWS) or, conversely, expression of several imprinted genes in chromosome 15q11–13) there is a single IC which has been removed by the inversion, remains monoallelic. While the IGFR2 methylated maternal allele of so does allelic methylation of IGFR2. The patients identified here with this type of mechanism carry a maternally inherited inversion of chromosome 11p with the breakpoint –300 kb centromeric (left) of IGFR2.

Figure 4. H19-dependent and -independent mechanisms lead to biallelic IGFR2 expression. The upper panel shows the normal allelic expression and methylation patterns of IGFR2 and H19. In BWS (lower panels), biallelic IGFR2 expression is observed in a majority of patients. In one class of patients, this is accompanied by hypermethylation of the maternal alleles of H19 and of IGFR2. In another class of patients examined here, H19 imprinting remains normal and so does allelic methylation of IGFR2. The patients identified here with this type of mechanism carry a maternally inherited inversion of chromosome 11p with the breakpoint –300 kb centromeric (left) of IGFR2.

It is of interest to compare the action of the imprinting mutation described here with those found in PWS/AS. Small deletions and other mutations upstream of the SNRPN gene have been found that either lead to a maternal epigenotype (methylation and expression of several imprinted genes in chromosomes 15q11–13) being present on a paternal chromosome (in PWS) or, conversely, a paternal epigenotype being present on a maternal chromosome (in AS). These mutations define an IC with possibly a bipartite structure, and it has been proposed that this IC is required in cis in the germlines to reset the grandparental epigenotypes (5,6). However, in the case of our inversion family, it is not obvious that there is a single IC which has been removed by the inversion, since H19 imprinting remains normal and allelic methylation in IGFR2 remains monoallelic. While the H19 locus can be considered a mini IC, there is nevertheless no genetic evidence thus far for a single IC in 11p15.5 that would control imprinting of all the genes in the domain.

The observation that replication timing was shifted to an earlier pattern by the inversion, yet H19 imprinting remained normal lends further support to our suggestion that the two phenomena can be genetically uncoupled (12). Hence, in the patients with a methylated maternal allele of H19, we found previously that replication timing remained entirely normal (12, Fig. 3). Although there was one case of an IC mutation in PWS/AS which seemed to result in an altered pattern of DNA replication (38), a more extensive analysis of several of these has now shown that there is little effect on replication asynchrony (39). This seems to bring our “methylated H19” patients more in line with the PWS/AS IC mutation cases.

The genetic evidence for an additional suppressor of IGFR2 obtained here is of interest with regard to evolutionary theories of imprinting. Hence, the genetic conflict theory of imprinting may suggest that an imprinted suppressor can spread more easily if it is tightly linked to a polymorphic imprinted gene with the opposite effect (40). The observation that there are at least two suppressors of IGFR2 (H19 and the locus identified by the inversion) could further suggest that a paternally expressed growth enhancer (IGFR2) could attract multiple linked maternal suppressors, which would be one explanation for the existence of imprinted gene clusters.

In conclusion, previous studies have shown that IGFR2 imprinting in a normal chromosome situation can be constrained by H19 through an enhancer sharing model. Here we have described the first imprinting mutation in BWS. This mutation leads to biallelic IGFR2 expression through an H19-independent mechanism, and shows that there is an imprinted suppressor locus of IGFR2 which is located centromerically to the IGFR2 gene (Fig. 4). Unravelling the mechanism by which mutations in this suppressor result in biallelic IGFR2 expression will give important insights into the pathogenesis of BWS, the regulation of IGFR2 imprinting, and into the many developmental and pathological situations in which there is biallelic expression of IGFR2 and monoallelic expression of H19, or vice versa.

MATERIALS AND METHODS

DNA and RNA methods and polymorphic markers

DNA was extracted from peripheral blood, and DNA methylation was analysed by restriction enzyme digest and Southern blotting as described (28). Primer amplification, DNA and RNA were extracted by ultracentrifugation through caesium trifluoracetate gradients as described (28), using 0.5 μg of genomic DNA and 1 μg of total RNA per reaction. Primers H1 and H3 were used to amplify the H19 3’-untranslated region (28), containing RsaI and TaqI polymorphisms (28). Primers IGFR2-08 and IGFR2-09 were used to amplify a part of the IGFR2 3’-untranslated region containing a CA repeat polymorphism (28) and primers P1 and P3 were used to amplify a fragment of the 3’-untranslated region (UTR) containing an ApoI polymorphism (28). Note that AvaiI and Apol detect the same polymorphism in exon 9 of IGFR2, but when AvaiI cuts, Apol does not, and vice versa. The nomenclature adopted in this paper is allele 1: AvaiI present, Apol absent; allele 2 AvaiI absence, Apol present.

PCR conditions for all sets of primers were as follows: 50°C for 60 min for the reverse transcription (for RNA only), followed by 3 min denaturation at 94°C, and then 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for both DNA and RNA. PCR products were diluted with appropriate buffers prior to digestion with RsaI, TaqI or Apol if required, and the products were separated on 1.5% agarose gels. For the IGFR2 CA repeat, one primer was end labelled with [γ-32P]ATP and the radiolabelled PCR products were separated on a non-denaturing 5% polyacrylamide gel (acrylamide:bis ratio 37.5:1) containing 5% glycerol and 0.5× Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.1). Gels were run at 18°C at 40 W for 4 h and bands detected on Hyperfilm β-max (Amersham).

All other polymorphic markers were used as described (12,28,36).
DNA replication analysis by FISH

Asynchronous cultures of lymphoblastoid cells were grown in RPMI supplemented with 10% fetal calf serum and 10^{-4} bromodeoxyuridine (BrDU) was added 90 min before harvest. Cells were harvested and fixed as described previously (31). The proportion of S phase cells was determined by immunofluorescence with anti-BrDU antibody conjugated to fluorescein isothiocyanate. Slides were hybridised to digoxigenin-labelled cINS containing the TH and INS genes and IGF2 up until exon 9. Detection, scoring and statistical analyses were done as described (41). The use of simultaneous BrDU detection was important since doublet signals can be found in some nuclei that are in G1.

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REFERENCES