Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms tumor

(imprinting/epigenotype/nephroblastoma/gene methylation)

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ABSTRACT  In most tissues IGF2 is expressed from the paternal allele while H19 is expressed from the maternal allele. We have previously shown that in some Wilms tumors the maternal IGF2 imprint is relaxed such that the gene is expressed biallelically. We have now investigated this subset of tumors further and found that biallelic expression of IGF2 was associated with undetectable or very low levels of H19 expression. The relaxation of IGF2 imprinting in Wilms tumors also involved a concomitant reversal in the patterns of DNA methylation of the maternally inherited IGF2 and H19 alleles. Furthermore, the only specific methylation changes that occurred in tumors with relaxation of IGF2 imprinting were solely restricted to the maternal IGF2 and H19 alleles. These data suggest that there has been an acquisition of a paternal epigenotype in these tumors as the result of a pathologic disruption in the normal imprinting of the IGF2 and H19 genes.

In humans, several genes are imprinted and monoallelically expressed (1, 2). Two of these genes, IGF2 and H19, are oppositely imprinted such that IGF2 is expressed from the paternal allele while H19 is expressed only from the maternal allele (3–7). IGF2 is an embryonal growth factor; however, the function of H19 is currently not understood, and although H19 RNA is abundantly transcribed in the embryo it does not appear to be translated (8). The expression patterns of IGF2 and H19 in differentiating human fetal tissues are similar (9, 10), and because of their close proximity at 11p15.5 (11), it has been proposed that these two genes may form an imprinted domain (1, 11–14).

Although the molecular basis of genomic imprinting is yet to be defined, allele-specific DNA methylation has been proposed as the most likely epigenetic mechanism (13, 15–17). Recently, we and others have found that in some Wilms tumors the transcriptionally silent maternal IGF2 allele is activated such that IGF2 expression occurs biallelically (3, 4). A similar biallelic pattern of IGF2 expression is also seen normally in the choroid plexus and leptomeninges, but both H19 alleles are silent (10). Conversely, in the methylase-deficient mouse, it has been shown that disruption of DNA methylation leads to the biallelic expression of H19 and an absence of IGF2 expression (14). Thus, DNA methylation appears to be essential to maintain the normal pattern of imprinting at the IGF2/H19 locus and it is possible that there is a common mechanism that regulates the reciprocal expression of these two genes. There is now evidence to suggest that H19 is an unusual class of tumor suppressor gene (18), which is silenced in some Wilms tumors by DNA methylation (19, 20). We have now investigated the relationship between the expression of IGF2 and H19 and the patterns of DNA methylation at the IGF2/H19 locus in three groups of Wilms tumors: those that show relaxation of imprinting and biallelically express IGF2, those that have a normal IGF2 imprint and express IGF2 monoallelically, and Wilms tumors that have lost heterozygosity at this locus.

MATERIALS AND METHODS

Sample Preparation, DNA/RNA Extraction, and Northern Blotting. Tumor samples and adjacent normal kidney were obtained at nephrectomy from Wilms tumor patients. Fetal kidney samples were obtained from aborted fetuses (12–17 weeks of gestation). DNA and RNA were extracted as described (3).

Allelic Analysis of IGF2 Expression. Allele-specific expression of IGF2 was determined as described (3).

Blotting Analysis. Northern blots were prepared as described (21). For DNA methylation analyses, Southern blots were prepared by digesting DNA (5 μg) with 50 units of Hpa II, Msp I, or Hha I; electrophoresed in 1.5% agarose gels; and blotted to Hybond N+ (Amersham). Filters were hybridized at 65°C with the following [32P]dCTP-labeled DNA probes: IGF2 (22), H19 (8), insulin (23), HRAS (24), and β-globin (25).

DNA Isolation PCR. DNA (10 μg) from tumors that expressed IGF2 monoallelically was digested with Hpa II and electrophoresed in 1.5% agarose. The methylated DNA fraction at ~3 kb (see Fig. 2a, lanes 8–12) was extracted from the gel and amplified by PCR with primers spanning the IGF2/Apa I or AC repeat polymorphisms (3, 4, 26). The PCR products were electrophoresed in 6% polyacrylamide gels after Apa I digestion to detect the Apa I polymorphism or directly to detect the AC repeat polymorphism.

RESULTS

IGF2 and H19 Expression in Wilms Tumors. Northern blots of a series of Wilms tumors showed that IGF2 was expressed at high levels in all 23 cases examined (Fig. 1a), as has been demonstrated (27, 28). Unlike IGF2, H19 RNA was detected in only 5/23 tumors, and all of these tumors expressed IGF2 monoallelically (Fig. 1b, lanes 8–12). In contrast to this group of tumors, the expression of H19 was undetectable or very low in 11 tumors that had undergone loss of heterozygosity (LOH) at 11p15 (Fig. 1b, lanes 13–23). These data are consistent with LOH studies in Wilms tumors that show a preferential loss of the maternal 11p allele (29–32), from which H19 is normally expressed (7). In a third group of tumors IGF2 was expressed biallelically and the levels of H19 RNA were also very low or undetectable (Fig. 1b, lanes 1–7). These data suggest that in these tumors an epigenetic change on the paternal chromosome has led to the activation of the maternal IGF2 allele and silencing of the maternal H19 allele.

Abbreviation: LOH, loss of heterozygosity.

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Methylation of IGF2 in Wilms Tumors with Normal IGF2 Imprinting. Methylation of IGF2 was examined with the methylation-sensitive restriction enzyme Hpa II or Hha I. When Southern blots were hybridized with an IGF2 cDNA probe that includes exons 3, 7, 8, and 9 (22), tumors that expressed IGF2 monoallelically showed a unique pattern of fragments of ~3 kb, which were resistant to both Hpa II (Fig. 2a, lanes 8–12, arrows) and Hha I (Fig. 2b, lanes 8–12, arrows) digestion. Similar methylation patterns were obtained for human fetal kidney (data not shown). Since this high molecular weight DNA fraction was not seen in tumors that had undergone maternal LOH (Fig. 2a and b, lanes 13–16), these methylated fragments were presumed to be derived from the maternal IGF2 allele.

To determine whether there was allele-specific methylation of IGF2, the Hpa II-resistant fragments in Fig. 2a (lanes 8–12) were isolated from an agarose gel and amplified by PCR using primers to detect the Apa I or AC repeat polymorphism. As shown in Fig. 3a (lanes 2, 4, and 6) and Fig. 3b (lanes 1 and 3), the Hpa II-resistant fragments were derived from one allele only. This finding contrasts with RNA PCR data, which showed that transcription occurred from the other IGF2 allele—that is, the allele that was not methylated (Fig. 3a, lanes 3, 5, and 7; Fig. 3b, lanes 2 and 4). Because IGF2 is transcribed from the paternal allele (3–5, 10), we conclude that the nonexpressed maternal IGF2 allele in these tumors was extensively methylated. This conclusion was formally verified in one case in which parental DNA was available. As shown in Fig. 3b, the methylated Hpa II-resistant fragments were derived from the upper allele (lane 3), while the expressed allele (lane 4) originated from the lower allele. A comparison of these data with the parental genotypes (lanes 6 and 7) demonstrated that IGF2 was expressed from the unmethylated paternal allele. In contrast to DNA methylation studies in the paternally disomic mouse (33), these studies show that in human kidney and Wilms tumors with paternal IGF2 expression the active paternal allele is unmethylated.

Methylation of IGF2 in Wilms Tumors with Relaxed IGF2 Imprinting. In seven Wilms tumors with biallelic IGF2 expression (Fig. 2a, lanes 1–7), Hpa II digestion resulted in a limited digest pattern (two fragments of 1.2 and 0.8 kb) corresponding to that seen with Msp I digestion (Fig. 2a, lanes 17–19). In contrast to the tumors that expressed IGF2 monoallelically, in these tumors the maternally derived IGF2 allele was absent (Fig. 2a, lanes 8–12). The same pattern was observed with Hha I digestion (Fig. 3b, lanes 1–7). These data indicate that there was extensive hypomethylation of both IGF2 alleles in tumors in which relaxation of IGF2 imprinting had occurred. Similarly, in tumors with maternal 11p LOH (Fig. 2a and b, lanes 13–16) and another seven tumors (data not shown), the absence of Hpa II- and Hha I-resistant fragments indicates that the retained IGF2 paternal allele was extensively hypomethylated.

Methylation of H19 Alleles in Wilms Tumor. The methylation status of the H19 alleles was also examined. The Southern blot that had previously been probed with an IGF2 cDNA probe (Fig. 2a) was stripped and reprobed with an H19 cDNA probe. As shown in Fig. 2c (lanes 8–12, arrows), low molecular weight fragments were present in DNA samples from tumors that expressed IGF2 monoallelically. Since these low molecular weight H19 fragments were not present in the DNA of tumors that had undergone maternal 11p LOH (Fig. 2c, lanes 13–16), this provides evidence that the inactive paternal allele of the H19 gene was methylated. A similar pattern of methylation was found in tumors in which H19 expression was absent and relaxation of IGF2 imprinting had occurred. As shown in Fig. 2c (lanes 1–7), both alleles of the inactive H19 gene were extensively methylated. These data are consistent with previous work, which showed that the nonexpressed paternal H19 allele is methylated (34).

Methylation of Other 11p15 Genes in Wilms Tumor. Because altered methylation of some 11p genes has been previously observed in human tumors and cell lines (35), we determined whether these changes were due to a generalized alteration in DNA methylation at 11p15 or confined to the IGF2/H19 locus. As shown in Fig. 4, after reprobing the Southern membranes used in Fig. 2 with a series of chromosome 11p probes, including insulin (Fig. 4a), HRAS (Fig. 4b),
IGF2 and H19 methylation in Wilms tumor. Genomic DNA from seven Wilms tumors with biallelic IGF2 expression (lanes 1–7), five with monoallelic IGF2 expression (lanes 8–12), and four with 11p LOH (lanes 13–16) were digested with Hpa II (a and c) or Hha I (b). Tumors with biallelic IGF2 expression (lane 17), monoallelic IGF2 expression (lane 18), and LOH (lane 19) were digested with Msp I (a and c) and then analyzed by Southern blotting with probes for IGF2 (a and b) or H19 (c). Molecular size was indicated as a HindIII/EcoRI-digested fragments in lane M.

and β-globin (data not shown), it is apparent that there was no consistent difference in methylation between tumors with or without relaxation of imprinting. These data indicate that in Wilms tumors with relaxed imprinting of IGF2, the changes in DNA methylation—i.e., the gain of methylation at H19 and the loss of methylation at IGF2—are confined to the IGF2/H19 locus.

**DISCUSSION**

A major finding of this paper is that relaxation of IGF2 imprinting in Wilms tumors is linked to specific alterations in the methylation patterns of both the IGF2 and H19 genes. These methylation changes affect the maternally inherited IGF2 and H19 genes such that there is a gain of methylation at H19 and a loss of methylation at IGF2. Concomitant with these methylation changes is a reversal in the normal pattern of gene expression such that the maternally inherited H19 and IGF2 alleles are silenced and activated, respectively.

Recent studies have shown that relaxation of IGF2 imprinting in Wilms tumor is associated with methylation of the H19 gene (19, 20), while here we report that methylation changes encompass the entire IGF2/H19 domain. More importantly, we have addressed the question of the specificity of methylation of these imprinted genes in Wilms tumor. DNA methylation changes at the IGF2 and H19 gene loci could reflect one of the two following situations: (i) the changes in DNA methylation were specific for the IGF2/H19 locus, or (ii) the methylation changes were simply a by-product of a generalized alteration in DNA methylation, which is characteristic of many tumors (36–38). We have shown that in all Wilms tumors, 11p15 genes were hypermethylated, regardless of the specificity of the methylation pattern at the IGF2/H19 locus. The specificity of the methylation changes of the IGF2 and H19 maternal alleles in tumors in which relaxation of IGF2 imprinting had occurred therefore provides evidence consistent with an epigenetic alteration at this locus being central to the tumorigenesis pathway.

Data suggesting that IGF2 is methylated in an allele-specific manner has previously been reported in one kidney sample;
Fig. 3. Correlation between expression and undermethylation of IGF2 (a and b) and parental origin of the methylated alleles (b) in Wilms tumors with monoallelic expression of IGF2 (a) Genomic DNA of case 8 (lane 1), DNA isolated from the 3-kb region in Fig. 2, lanes 8, 10, and 12 (lanes 2, 4, and 6, and the corresponding cDNA of cases 8, 10, and 12 (lanes 3, 5, and 7) were amplified by PCR with a set of primers for Ape I polymorphism. (b) Genomic DNA of case 11 (lane 5), DNA from the 3-kb region in Fig. 2, lanes 9 and 11 (lanes 1 and 3), cDNA of cases 9 and 11 (lanes 2 and 4), and genomic DNA of the father (lane 6) and mother (lane 7) of case 11 were amplified with a set of primers for AC repeat polymorphism.

However, the parental origin of methylation and IGF2 imprinting status were not determined (39). Recently, it was found that a region in exon 9 of the human IGF2 gene is specifically methylated on the paternal allele in peripheral blood and in a variety of tissues from patients with the Beckwith-Wiedemann syndrome (40), but we have been unable to demonstrate a similar pattern of methylation at this site in normal kidney and Wilms tumors (M.J.S., unpublished data). The epigenetic modification of the IGF2 locus that we have detected in Wilms tumors and kidney is not present in all tissues and therefore provides an example of a tissue-specific methylation imprint that is unrelated to gamete-of-origin imprinting. In contrast to these findings, it has been reported that in mouse embryos there is a region of methylation upstream from the paternal IGF2 allele, although exons of both alleles are largely unmethylated (33). The differences in methylation seen in these mouse tissues and in human kidney and Wilms tumors may be due to variations in tissue-specific methylation imprinting.

IGF2 is expressed monoallelically in most tissues; however, biallelic expression is found in the choroid plexus and leptomeninges where there is also no expression of H19 (10, 41, 42). This finding, together with the close proximity of IGF2 and H19, has led to the suggestion that IGF2 and H19 form a coordinately regulated imprint domain, which may be controlled by a bidirectionally acting H19 enhancer (1, 11–14). The question arises as to the mechanism of the epigenetic changes that lead to relaxation of IGF2 imprinting. Parallels with the XIST gene may provide clues to understanding the events leading to the epigenetic changes at the IGF2/H19 locus. For example, H19 and XIST both act in cis, neither of the RNA products appears to be translated, and both genes lie within imprinted regions of the genome (43, 44). By analogy with XIST and chromosome X inactivation, the function of H19 may be required to initiate events that lead to inactivation of the IGF2/H19 locus. Accordingly, data shown here which demonstrate that methylation of the human IGF2 gene occurs in a tissue-specific fashion and equates to gene silencing are consistent with IGF2 methylation being secondary to the events that initiate the allele-specific repression of IGF2 transcription.

That H19 may be the primary imprinted gene, the activity of which is required to suppress IGF2 transcription in cis, has previously been suggested to explain the patterns of H19 and IGF2 expression in methylese-deficient mice (14). Experiments reported here are consistent with the notion that relaxation of IGF2 imprinting in Wilms tumor may be initiated by a pathologic methylation and silencing of the maternal H19 allele, which in turn leads to loss of methylation and activation of the maternal IGF2 allele as a secondary event. In support of this model, we have shown that in all Wilms tumors examined, genes within 11p15 were consistently methylated, suggesting that relaxation of IGF2 imprinting could have arisen by an aberrant H19 methylation. This model makes several predictions. For example, the constitutional relaxation of IGF2 imprinting observed in some children with somatic overgrowth and predisposition to Wilms tumor (45, 46) may sometimes be associated with aberrant methylation and silencing of the actively transcribed maternal H19 allele. In support of this possibility, we have recently shown that in one patient with gigantism and Wilms tumor, both H19 alleles were constitutionally methylated and IGF2 was transcribed biallelically (T.T., unpublished data). Similarities to this situation may be forthcoming from H19 null mice. It will be intriguing to determine whether the germ-line inactivation of the maternal H19 gene in mice can lead to a growth-enhanced phenotype similar to the Beckwith-Wiedemann syndrome.

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