Methylation Sequencing Analysis Refines the Region of H19 Epimutation in Wilms Tumor*

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Differential DNA methylation of the parental alleles has been implicated in the establishment and maintenance of the monoallelic expression of imprinted genes. H19 and IGF2 are oppositely imprinted with only the maternal and the paternal alleles expressed, respectively. In Wilms tumor, a childhood renal neoplasm, loss of the H19/IGF2 imprinted expression pattern results in silencing of H19 and biallelic expression of IGF2. This was shown to be associated with biallelic methylation of the H19 promoter in the tumor and the adjacent kidney tissue suggesting that epigenetic H19 silencing is an early event in Wilms tumorigenesis. An imprinting mark region characterized by paternal allele-specific methylation has been suggested to reside in a GC-rich region of 400-base pair repeats starting at −2 kilobase pairs (kb) relative to the H19 transcription start and extending upstream. The upstream boundary of the potential paternal methylation imprint of the H19 gene has yet to be defined. We sought to define this upstream imprint boundary and investigate whether Wilms tumors with loss of imprinting are biallelically methylated in this imprinting mark region. The analysis of 6.8 kb of new upstream H19 sequence determined in this study identified a series of the direct 400-base pair repeats that extends to approximately −5.3 kb relative to the transcription start. DNA methylation analyses indicated that the upstream boundary of the potential imprint may coincide with the 5′ end of the direct repeats. We found that Wilms tumors with loss of imprinting are biallelically methylated in the H19 upstream repeat region, and we suggest that pathological methylation in this region is the epigenetic error that initiates H19 silencing.

Genomic imprinting describes the phenomenon of heritable parent-of-origin-specific expression of genes. The molecular mechanisms that determine the monoallelic expression of imprinted genes are to date not fully understood. However, it is a widely accepted concept that parental allele-specific DNA methylation plays an important role in the process. The insulin-like growth factor 2 (IGF2) and H19 genes are located in a cluster of imprinted genes on human chromosome 11p15.5 that is syntenic with the mouse distal chromosome 7. The maternally imprinted IGF2 gene is transcribed only from the paternal allele in most normal human tissues except for adult liver, choroid plexus, and leptomeninges (1, 2). In contrast, the H19 gene is paternally imprinted hence maternally silenced (3–5). This reciprocal expression pattern is frequently lost in Wilms tumor, a childhood renal neoplasm, and in the overgrowth syndrome, Beckwith-Wiedemann syndrome, that predisposes to Wilms tumor either by maternal loss of heterozygosity at chromosome 11p15 or by loss of imprinting (LOI) (6). LOI of IGF2 in Wilms tumor was first described by Rainier et al. (7) and Ogawa et al. (8). Since then several groups have shown that LOI of IGF2 in Wilms tumor and Beckwith-Wiedemann syndrome is associated with transcriptional repression and hypermethylation of the maternal H19 allele (9–14). These findings led to the hypothesis that early in embryonal development the maternal allele of H19 becomes silenced by DNA methylation which then relaxes maternal IGF2 silencing in cis thereby conferring a growth advantage to the affected cell. Strong evidence supporting this idea came from mouse studies in which biallelic Igf2 expression was observed following maternal transmission of deletions at the H19 locus (15, 16). More recently Cui et al. (17) have reported that loss of H19 expression occurs frequently in Wilms tumors and their precursor lesions irrespective of whether or not IGF2 is mono- or biallelically expressed. At present, it is therefore not clear whether loss of H19 and gain of IGF2 expression from the maternal allele in Wilms tumors are independent processes or not.

Differential DNA methylation of particular sites or regions is thought to provide an epigenetic or imprinting mark that distinguishes the parental alleles of imprinted genes (18). DNA methylation analyses of the mouse H19 gene have identified a region of paternal allele-specific methylation that is maintained throughout murine development and may therefore function as an imprinting mark (19, 20). A genomic deletion of 1.6 kb within this region has been shown to abrogate allele-specific H19 and Igf2 expression highlighting the importance of this region in H19/Igf2 imprinting control (21). The human H19 gene and its 5′-flanking region are also methylated on the silent paternal allele, whereas the maternal allele is unmethylated (22, 23). A candidate region for a paternal methylation imprint of the human H19 gene has been proposed that contains several unique 400-bp direct repeat sequences starting at −2 kb and extending further upstream (23). A number of HpaII and HhaI sites in this region were shown to be methylated in sperm and on the paternal alleles in somatic cells, and this...
methylated was found to be preserved in pooled 8–32 cell stage embryos. An evolutionary conserved sequence within these potential imprinting mark regions of mouse and human which may be involved in the control of H19 and IGF2 imprinting was recently identified in our laboratory (24).

Previous investigations of H19 methylation in Wilms tumor examined only the promoter, gene body, and 3’ flank of H19, thereby solely relying on the usage of methylation-sensitive restriction enzymes. The potential imprinting mark region has not yet been investigated in Wilms tumors. In the present study we have therefore analyzed H19 methylation in Wilms tumors at several new upstream locations including the imprinting mark and the promoter region using Southern blotting and bisulfite genomic sequencing. Our analysis delineated the upstream boundary of the imprinting mark to approximately −5.3 kb. The bisulfite genomic sequencing analysis revealed highly variable methylation patterns in the H19 promoter region in Wilms tumors and adjacent kidneys regardless of their imprinting status. In contrast, the potential imprinting mark region showed very consistent paternal specific methylation. Wilms tumors with LOI were hypermethylated at the imprinting mark and the promoter region using Southern blotting and bisulfite genomic sequencing. Our analysis delineated the upstream boundary of the imprinting mark to approximately −6 kb. The bisulfite genomic sequencing analysis revealed highly variable methylation patterns in the H19 promoter region in Wilms tumors and adjacent kidneys regardless of their imprinting status. In contrast, the potential imprinting mark region showed very consistent paternal specific methylation. Wilms tumors with LOI were hypermethylated at the imprinting mark region consistent with the hypothesis that an epigenetic error in this region may initiate H19 silencing.

EXPERIMENTAL PROCEDURES

Sequence Data—The upstream H19 sequence used in this study is available from GenBank™ accession number AF125183.

Wilms Tumors IGF2 Allelic Analysis—The Wilms tumors used in this study were previously typed in our laboratory for 11p15 LOH using polymorphic markers in the H19/IGF2 region. In non-LOH cases monor biallelic expression of IGF2 was determined as described (8).

Southern Blot Analysis—Digests were performed with Rsal (8 units/μg), MspI (10 units/μg), and HpaII (13 units/μg). Southern blots contained 6 μg of DNA per lane. Hybridization was at 65 °C for 16 h. Probes were obtained from plasmid clones containing H19 5’ sequence by restriction digest and purification of the appropriate size fragment. To control for incomplete HpaII digestion, the Southern blots were hybridized with a probe for mitochondrial DNA. All samples showed a single HpaII fragment indicating complete digestion (data not shown).

Bisulfite Treatment—Prior to bisulfite treatment 4 μg of genomic DNA were resuspended in 16 μl at 37 °C (40 units) or with PstI (40 units) or with HpaII (40 units) in combination. The digested DNA was ethanol-purified and resuspended in 40 μl of H₂O. The bisulfite treatment was carried out as described by Clark et al. (25) with the following alterations. The bisulfite reaction under mineral oil was performed at 60 °C for 16 h in 525 μl of total volume containing 2.4 mM sodium bisulfite (Sigma) and 123 mM hydroquinone (Sigma). Reactions were desalted using the QIAEX II gel extraction kit (Qiagen). The DNA was eluted in 50 μl of H₂O, incubated with 5 μl of 3 M NaOH for 15 min at 37 °C, neutralized with ammonium acetate (final concentration of 3 M), and ethanol-purified. The bisulfite-treated DNA was then resuspended in 50 μl of H₂O and stored at −20 °C.

PCR—To obtain the PCR products bis1 and bis2, nested PCRs were performed using 5 μl of bisulfite-treated DNA in the first amplification (25 μl total volume) and 5 μl of this PCR product as template in the second amplification (50 μl total volume). All reactions contained 0.6 μl of primers, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.25 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). PWO DNA polymerase (1 units) was added during the second amplification to obtain blunt-ended product for DNA cloning. The following primer pairs were used under the given conditions. For bis1, outer primers 1133 (5’-TGATGTTGTAAGGAGGTTTTTGGTTT and 1134 (5’-CTCC-CTCCACAACCCCATCTCCTCCCATATTA), 0.75 μM MgCl₂, 57 °C annealing temperature (AT); inner primers 1143 (5’-GGAAGTTGGAAGGTGTTT and 1144 (5’-CATCACCACCCCTCCCTCACCAT), 1 μM MgCl₂, 53 °C AT. For bis2, outer primers 1093 (5’-GGGTGGTTGGAAGGTGTTT and 1094 (5’-CCCAACTTATTTAACAACTCAG), inner primers 1096 (5’-GGGTTTGGTTGGAAGGTGTTT and 1097 (5’-ACTAAAAACACATCACAAGTTT), both pairs at 2 mM MgCl₂ and 50 °C AT. The PCRs were performed on a Peltier Thermal Cycler-200 (MJ Research) using the following programs: first round of amplification: 1 time at 95 °C for 13 min, 5 times at 94 °C for 1 min, AT for 2 min, 72 °C for 3 min, and 25 times at 94 °C for 30 s, AT for 2 min, 72 °C for 1.5 min, and 1 time 72 °C for 6 min; second round of amplification: 1 time at 95 °C for 13 min, 5 times at 94 °C for 1 min, AT for 2 min, 72 °C for 3 min, 18 times at 94 °C for 30 s, AT for 2 min, 72 °C for 1.5 min, hold on 4 °C to add 1 unit of PWO DNA Polymerase to every reaction, 10 times at 95 °C for 30 s, AT for 2 min, 72 °C for 1.5 min, and 1 times 72 °C for 6 min.

Cloning of PCR Products The PCR products bis1 and bis2 obtained from the second round of amplification were gel-purified using the QIAquick gel extraction kit (Qiagen). Purified fragments were cloned into the EcoRV site of the pBluescript SK− vector. Ligation were for 12 h at 16 °C and contained circular pBluescript:insert at a molar ratio of 12:1, 200 units of T4 DNA ligase (New England Biolabs), 20 units of EcoRV, and ligation buffer (New England Biolabs) in 10 μl total volume.

DNA Sequencing—Plasmids were sequenced with the T3 primer using the ABI sequencing system (Perkin-Elmer Applied Biosystems).

RESULTS

Analysis of H19 Upstream Sequence—The previous analysis of 3.4 kb of H19 upstream sequence by Jinno et al. (23) identified a 400-bp direct repeat that is reiterated 3.5 times. These repeats were suggested to harbor the paternal methylation imprinting mark of the human H19 gene. To determine the extent of this repeat motif, we obtained an additional 6.6 kb of sequence 5’ of H19 (Fig. 1, GenBank™ accession number AF125183). The subsequent analysis of the resulting 10 kb of 5’ H19 sequence identified two repeat units (1 and 2) that each consist of an H19 proximal 450-bp direct repeat (A1 and A2) followed by several 400-bp repeats (B1–7), two of which are incomplete (B4 and B7). Repeat Unit 2 extends to −5.3 kb upstream of the H19 transcription start and is separated from Repeat Unit 1 by 387 bp of unique sequence. The 450-bp motifs A1 and A2 are 85.4% identical. The 400-bp motifs B1, B2, and B3 of Repeat Unit 1 as well as B5 and B6 of Repeat Unit 2 show a remarkable 85–91% identity to each other. Further upstream we found two complete Alu elements, one incomplete retroviral long terminal repeat, four MLTI elements, and a 29-bp GC-rich repeat.

Methylation Analysis of H19 Upstream Regions in Wilms Tumor—If an epigenetic error was the cause of H19 silencing in Wilms tumor, then this error would most likely occur in the potential imprinting mark region that controls the establishment of imprinted H19 expression. The maternal hypermethylation found at the H19 promoter and sequences further downstream might then be a secondary event that occurs after the maternal allele has acquired a paternal epigenotype at the imprinting mark. The first objective of this study was to test this hypothesis by investigating the allele-specific methylation in the respective H19 upstream regions. We examined pairs of tumor and corresponding normal kidney tissue samples from Wilms tumor patients that have retention of IGF2 imprinting (ROI), loss of IGF2 imprinting or maternal loss of chromosome 11p15.5 (LOH) in the tumor tissue, and a sperm DNA sample from a healthy donor. The bisulfite genomic sequencing method (26) was used to analyze a 327-bp fragment (bis1) in the H19 promoter region and a second fragment (bis2) of 504 bp located within the potential imprinting mark region (Fig. 1). Polymorphisms were identified within both fragments, which allowed us to distinguish between maternal and paternal alleles in heterozygous samples.

A second objective of this study was to find the upstream boundary of differential H19 methylation. To do this Southern blots of Rsal/HpaII double-digested Wilms tumor, corresponding kidney, and an sperm DNA sample hybridized to the four 5’ H19 probes indicated in Fig. 1. Regions of differential methylation were identified in this analysis by comparing the intensity ratio of a larger Rsal fragment (derived from methylated maternal alleles) to a smaller HpaII fragment (derived from unmethylated maternal alleles) in kidney tissue and ROI Wilms tumor samples. Wilms tumors with maternal 11p15 LOH showed only
the paternal methylation pattern, whereas LOI Wilms tumors with hypermethylation of the 5' H19 region displayed a shift in signal intensity toward the larger Rsal fragment that was proportional to the number of cells in the sample that are methylated at the addressed HpaII sites on the maternal alleles. The methylation data obtained in the Southern blotting analysis have been summarized in Table I.

**Analysis of the Promoter Region by Southern Blotting (Probe 1)—**Southern blots were hybridized with probe 1 to determine the methylation status of HpaII sites 43 and 44 (H43 and H44 in Fig. 1) in the promoter region (blots in Fig. 2 and the legend in Table I). The Rsal/HpaII-digested samples showed three prominent fragments (indicated R7-R8, R7-H46, and H43-H44 in Fig. 2, a and b). Fragments R7-R8 and R7-H46 corresponding in size to the respective restriction digest fragments (Fig. 1) were of paternal origin since they appeared in all tumor and kidney samples including the LOH cases. This indicated that HpaII site 46 (H46) was paternally unmethylated in a high proportion of cells in Wilms tumors and in kidneys regardless of the IGF2/H19 imprinting status. We can therefore conclude that H46 does not show strict paternal-specific methylation.

The five ROI Wilms tumors and the corresponding normal kidney tissues showed the expected 1:1 ratio of the maternal fragment H43-H44 (Fig. 2a) compared with the paternal fragments R7-R8 and R7-H46. In contrast, four of six LOH Wilms tumors lacked the HpaII fragment indicating that the paternal alleles were methylated at sites H43 and H44 (Fig. 2, a and b) in agreement with previous reports (9, 14). Surprisingly, LOH Wilms tumors 47 and 55 displayed weak HpaII fragments (Fig. 2b) indicating that the paternal alleles were unmethylated at H43 and H44 in a minor proportion of cells in these tumors. This was unlikely to be caused by contaminating normal tissue considering the high purity of the tumors.

The LOI Wilms tumors were hypermethylated to different extents in the promoter region (Fig. 2b). HpaII fragments were absent in LOI cases 30, 31, and 65, hence both parental alleles were fully methylated (except H46 as described above) (Fig. 2b). The corresponding kidneys also contained a high proportion of hypermethylated maternal alleles implied by the stronger intensity of fragments R7-R8 and R7-H46 compared with H43-H44 (Fig. 2b). Hypermethylation in the adjacent kidney tissue of LOI Wilms tumors has previously been shown and has led to the conclusion that epigenetic changes at the H19 locus are an early event in Wilms tumorigenesis (14). The LOI Wilms tumors 43 and 49, however, were hypermethylated to a lesser extent at H43 and H44 indicated by the presence of HpaII fragments of different intensities (Fig. 2b). Sperm DNA (Fig. 2a) was unmethylated at both sites consistent with previous reports (9).

**Analysis of the Promoter Region by Bisulfite Sequencing (bis1)—**The bisulfite genomic sequencing analysis of the 12 CpGs closest to the H19 transcription start (bis1, Fig. 1) gave detailed methylation patterns from single clones of paternal and maternal alleles (Fig. 3). Sperm DNA was unmethylated at nearly all 12 CpGs concordant with the Southern blotting results for H43 and H44.

The predominant observation for all Wilms tumors and kidneys examined was the variability of methylation patterns in the H19 promoter. Although paternal alleles were significantly more methylated than maternal alleles, none of the 12 CpGs examined was found to be consistently methylated on all paternal or unmethylated on all maternal alleles. The extent to which the paternal alleles were methylated varied from 1/12 to 12/12 CpGs. Equally surprising were the maternal methylation patterns in that the presumably normal maternal alleles of normal kidneys and ROI Wilms tumors that were expected to be fully unmethylated also harbored methylation. This was particularly obvious for heterozygous ROI Wilms tumor and kidney 110 that contained up to 7 methylated CpGs in a single maternal allele.

LOI Wilms tumors 30, 31, and 43 showed hypermethylation of the maternal alleles consistent with previous methylation

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**Fig. 1. Model of 10 kb of upstream H19 sequence and bisulfite PCR fragments.** a, on the sequence line are depicted the H19 transcription start site (arrow), HpaII sites (short vertical lines), and Rsal sites (longer vertical lines numbered R1-R8). HpaII sites that were addressed in the methylation analysis are boxed and numbered (e.g. H46). Southern blotting probes 1–4 are indicated above the sequence line. Note that probe 2 hybridizes at two locations due to high sequence homology of repeats A1 and A2. Repetitive sequence elements are indicated below the sequence line. Repeat Units 1 and 2 consist of homologous 450-bp direct repeats A1 and A2, and several 400-bp direct repeats B1–7. B4 and B7 are incomplete and contain only 130 and 250 bp of homologous sequence, respectively. The locations of the PCR fragments bis1 and bis2 generated in the bisulfite genomic sequencing analysis are indicated below the sequence line. b, fragments bis1 and bis2 are shown enlarged (the 100-bp scale bar applies). The short vertical lines on bis1 and bis2 depict all CpGs found in these fragments. Every fifth CpG within each fragment is numbered. CpGs that coincide with an HpaII site are marked below according to the nomenclature used in a. The arrows below bis1 and bis2 indicate the location of single base pair polymorphisms (in 5' to 3' order: bis2, C/T, CA, G/A, G/C, and C/T; bis1, G/A).
analyses at HpaII sites. The corresponding kidneys also contained hypermethylated maternal alleles consistent with a previous study from our laboratory (14). However, the extent and distribution of methylation on the paternal and maternal alleles was strikingly variable within the same LOI case, as well as between the three LOI cases. The fact that the methylation patterns in the H19 promoter region were highly variable may argue against a causative role of methylation in this region in the establishment or maintenance of H19 silencing.

Analysis of the Upstream Repeat Region by Southern Blotting (Probe 2)—The Southern blotting analysis with probe 2 (Fig. 4 a and b) gave a complex fragment pattern indicating the methylation status of HpaII sites 25, 34, and 35 (H25, H34, and H35) since this probe hybridized simultaneously to two locations, repeat A1 and A2 (Fig. 1). These HpaII sites are located within the upstream repeat region that was suggested to function as the human H19 imprinting mark (23).

The methylation status of H34 and H35 was examined using probe 2 that hybridized to repeat A1. The respective Southern blots showed the RsaI fragment R6-R7 from the paternal alleles and the HpaII/RsaI fragments H34-R7 and H35-R7 from the maternal alleles (Fig. 4, a and b). This interpretation of the parental origin of the RsoI and HpaII fragments is supported by the previously reported paternal allele-specific methylation for this region (23) and has been verified by our own bisulfite genomic sequencing results (Fig. 5). Analysis of the kidney and tumor tissues of ROI cases showed two maternally derived genomic sequencing results (Fig. 5). Analysis of the kidney and tumor tissues of ROI cases showed two maternally derived genomic sequencing results (Fig. 5).

**TABLE I**

Summary of H19 methylation analysis in Wilms tumor, adjacent kidney, and sperm

| H15 | H16 | H17 | H18 | M1* | H22 | H23 | M1* | H25 | M1* | H24 | H25 | H26 | H27 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 4a  | 3a  | 2   | 2   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   | 1   |
| ROI-WT and adjacent K | | | | | | | | | | | | | |
| 70T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 106T| +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 109T| +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 110T| +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 117T| +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| LOI-WT and adjacent K | | | | | | | | | | | | | |
| 30T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 31T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 45T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 49T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 65T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| LOH-WT and adjacent K | | | | | | | | | | | | | |
| 4T  | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 55T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 84T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 86T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 88T | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| K   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| 100T| +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   | +   |
| Sperm| + | + | + | + | + | + | + | + | + | + | + | + | + |

a Signal intensities on Southern blots were also quantified by densitometry to calculate a Methylation Index (MI) for each sample and probe. MI equals the RsoI band intensity over the sum of HpaII-band intensities for each lane (hypomethylation, MI <0.7; differential methylation, 0.7> MI >1.5; hypermethylation, MI >1.5).

b Site and probe nomenclature corresponds to Fig. 1. Signals for probes 1 and 2 were scored from blots shown in Figs. 2 and 4, respectively. Blots hybridized with probes 3 and 4 are not shown.
Methylation Sequencing Analysis of 5' H19 Regions

The LOH Wilms tumors showed different fragment patterns for the potential imprinting mark region. HpaII fragments H34-R7 and H35-R7 were absent in LOH tumors 86 and 88, but both cases displayed the fragment H32-R7 (Fig. 4a, lanes 86T and 88T). This indicated paternal methylation at H34 and H35 and a considerable cell population being unmethylated at H32. LOH Wilms tumors 100, 47, and 55 displayed weak signals for H34-R7 and H35-R7 (Fig. 4b, lane 47T) indicating that these LOH tumors were paternally unmethylated at these sites in a minor proportion of cells. LOI Wilms tumors 30 and 31 also contained methylated maternal alleles.

For the LOI Wilms tumors 30, 31, and 43 that showed hypermethylation to different extents in the upstream repeat regions, although highly variable fragment patterns and different fragment intensities for the addressed HpaII sites suggested that maternal methylation is not complete in these tumors and seems to be of different distribution comparing the LOI cases with each other. It is interesting to note that patients 31 and 65 whose LOI Wilms tumors showed the highest degree (nearly 100%) of maternal hypermethylation in the potential imprinting mark region were patients with systemic overgrowth phenotype (case 31 had hemihypertrophy and multiple WT; case 65 corresponds to case 1 in Morison et al. (27)).

Analysis of the Upstream Repeat Region by Bisulfite Sequencing (bis2)—Using the bisulfite genomic sequencing method we analyzed 27 CpGs within Repeat Unit 1 (bis2, Fig. 1). The detailed methylation patterns from single clones of paternal and maternal alleles are shown in Fig. 5. Sperm DNA was highly methylated with the number of methylated sites varying between 21 and 26 of the 27 CpGs. In samples with normal IGF2/H19 imprinting (106, 110, 134, and kidney 55) the CpGs 1–20 of bis2 that map to the 400-bp direct repeat A1 showed very consistent paternal-specific methylation. These findings confirm that the 400-bp direct repeats are likely to harbor the paternal methylation imprint of H19. In contrast, some of the CpGs (numbers 21–27) that are located within the 450-bp direct repeat A1 showed biallelic methylation in the kidney and tumor samples (except ROI-WT 110, Fig. 5). This variable biallelic methylation at CpGs 21–27 indicated that the A1 repeat sequence itself is not part of the methylation imprinting mark. On the contrary, the A1 repeat may constitute the 3' boundary of the imprint that therefore may reside solely within the 400-bp direct repeats (B1-B7).

For the LOI Wilms tumors 30, 31, and 43 that showed hypermethylation to different extents in the upstream repeats on Southern blots (as described above, Fig. 2 and Table I), we obtained more detailed methylation patterns of both parental alleles for the bis2 fragment (Fig. 5). LOI tumors 30 and 31 showed nearly identical patterns of hypermethylation. In LOI tumors 30 and 31, 98% of the CpGs within bis2 were methylated on both parental alleles. Hence, for these two tumors the methylation patterns of the maternal and paternal alleles were practically identical in this region. LOI tumor 43, however, contained normal unmethylated (numbers 13–27) as well as methylated (numbers 1–12) maternal alleles. The number of methylated CpGs ranged from 0 to 27 of 27 sites, and methylation was distributed randomly over the 27 CpGs. Consequently, the methylation patterns of hypermethylated maternal alleles were significantly different from those of the paternal alleles that displayed the expected high density of methylation. The adjacent kidney tissues of the LOI Wilms tumors 30 and 31 also contained methylated maternal alleles and were therefore mosaic for H19 methylation in the upstream repeat region as was shown for the promoter region. The kidney of LOI case 43 gave only one partly hypermethylated cell being hypermethylated in the H19 upstream repeat region. Kidney 31 was hypermethylated to a lesser extent indicated by stronger RsaI/HpaII fragment intensities. LOI Wilms tumors 30, 43, and 49 and the adjacent kidneys displayed different fragment patterns. Tumor 30 showed strong hypermethylation for H34 and H35 but no detectable hypermethylation for H25 (Fig. 4b, lane 30T). The corresponding kidney 30 showed a lower degree of hypermethylation at H34 and H35 (Fig. 4b, lane 30K). LOI Wilms tumors 43 and 49 showed hypermethylation for H25, H34, and H35 compared with the corresponding kidneys, however of much lesser extent than the other LOI cases (Fig. 4b, lanes 43T, K and 49T, K). In summary, the LOI Wilms tumors were hypermethylated in the upstream repeat regions, although highly variable fragment patterns and different fragment intensities for the addressed HpaII sites suggested that maternal methylation is not complete in these tumors and seems to be of different distribution comparing the LOI cases with each other. It is interesting to note that patients 31 and 65 whose LOI Wilms tumors showed the highest degree (nearly 100%) of maternal hypermethylation in the potential imprinting mark region were patients with systemic overgrowth phenotype (case 31 had hemihypertrophy and multiple WT; case 65 corresponds to case 1 in Morison et al. (27)).
FIG. 3. Bisulfite genomic sequencing analysis of the H19 promoter region. Genomic DNAs from Wilms tumors (WT), adjacent kidneys, and sperm were bisulfite-treated, PCR-amplified, and single clones sequenced to give the methylation pattern of 12 CpGs located in the H19 promoter region (bis1 PCR depicted in Fig. 1; + for methylated, − for unmethylated). The tumors differed in the IGF2 imprinting status. ROI-WTs 110 and 106 have retained IGF2 imprinting, LOI-WTs 30, 31, and 43 express IGF2 biallelically, and LOH-WT 55 has maternal 11p15.5 chromosome loss. Sperm DNA from a healthy donor was also analyzed. Clones are numbered and marked with an asterisk, if they were obtained upon digestion with HpaII prior to bisulfite treatment (see “Experimental Procedures”). This predigest was necessary to overcome a PCR bias that favored the amplification of unmethylated alleles. For samples heterozygous at a G/A-polymorphism (depicted in Fig. 1), the parental origin of alleles was identified, and the clones were grouped into paternal and maternal alleles.
Methylation Analysis of Regions 5’ to the 400-bp Repeats—To address the question whether the paternal-specific methylation extends 5’ of Repeat Unit 2, we analyzed the methylation status of HpaII sites 22 and 23 (H22 and H23) and 15–18 (H15–H18) by Southern blotting using probes 3 and 4, respectively (Fig. 1). The methylation data have been summarized in Table I (Southern blots are not shown). Sites H22, H23, H17, and H18 that are located within Alu elements (Alu-Sx and Alu-Y, Fig. 1) did not exhibit differential methylation. Instead, all Wilms tumors and kidney samples were highly methylated at these sites, except ROI Wilms tumor 110 that was fully unmethylated within the Alu sequences. The methylated state of these HpaII sites in most samples corresponded well with reports of Alu repeats being highly methylated in somatic tissues (28, 29). The hypomethylation observed for Wilms tumor 110 might indicate that this tumor has lost the ability to methylate Alu elements. HpaII sites 15 and 16 located approximately 7.8 kb upstream of H19 (Fig. 1) were less than 50% methylated in kidney samples and are therefore unlikely to be differentially methylated. However, all Wilms tumors including the ROI cases (except tumor 110) showed hypermethylation to different extents at H15 and H16 compared with the corresponding kidneys. In summary, hybridization probes 3 and 4 revealed methylation patterns further 5’ to the 400-bp direct repeats upstream of H19 that were inconsistent with paternal-specific methylation.

In conclusion, this DNA methylation analysis suggests that the region of paternal allele-specific methylation 5’ of H19 that is also methylated in sperm might be confined to the 400-bp direct repeats that extend from −2 to −5.3 kb relative to the H19 transcription start site. We have also shown that this region is hypermethylated to different extents on maternal alleles in Wilms tumors with loss of IGF2 imprinting and, furthermore, that the pathological methylation is more pronounced in the upstream repeats compared with the H19 promoter region.

DISCUSSION

This investigation is the first to exploit the bisulfite genomic sequencing method to investigate H19 methylation in humans. Combining this method with results from methylation-dependent Southern blotting, we describe a detailed investigation of methylation patterns of the H19 promoter and regions upstream in Wilms tumor. Abnormal methylation of the H19 locus is important in the pathogenesis of Wilms tumor and the fetal overgrowth syndrome, Beckwith-Wiedemann syndrome (BWS). An H19/Igf2 imprinting control region that harbors a paternal methylation imprint as determined by bisulfite genomic sequencing has been identified in the mouse, which upon genomic deletion led to H19 silencing and biallelic Igf2 expression (20, 21). For humans an equivalent methylation imprint was suggested to be located at a similar H19 upstream position (22). However, H19 methylation within this upstream region that seems to be critical for H19 and IGF2 imprinting had not been studied in Wilms tumor prior to this investigation.

Previous studies have shown that pathological methylation of maternal alleles in the H19 promoter is linked to loss of IGF2 imprinting in Wilms tumor and cases of BWS based on the analysis of five HpaII sites in this region (designated 42–46 in this study) (9–11, 14, 27). Our investigation initially confirmed by Southern blotting that HpaII sites 43 and 44 in the promoter region are hypermethylated in LOI Wilms tumors. However, our results demonstrated that the extent of hypermethylation
at these sites differed between the LOI tumors. For HpaII site 46 in the H19 promoter, we found a lack of differential methylation in all Wilms tumors and kidney samples regardless of their imprinting status. Bisulfite sequencing analysis subsequently showed that the pathological H19 promoter methylation of maternal alleles in LOI Wilms tumors is highly variable in extent and distribution. Furthermore, in tumors and kidneys with normal IGF2 imprinting, the 12 CpGs in the repeat displayed only a low degree of differential methylation. Although paternal alleles were generally more methylated than maternal alleles, the number of CpGs methylated on any paternal allele was found to be highly variable, and the distribution of methylation appeared to be completely random. In addition, some maternal alleles from tumors and kidneys with normal imprinting showed a considerable number of methylated CpGs.

These results are of particular interest in view of other studies that have used HpaII site 46 to examine changes in parental allele-specific methylation at the H19 locus in BWS patients (11, 13, 30). These studies may potentially underestimate the proportion of BWS cases that harbor hypermethylated H19 by examining only this one site. In conclusion, the heterogeneity of methylation at the H19 promoter indicated to us that this region is inappropriate for future investigations into the pathological hypermethylation of the H19 gene in Wilms tumor and BWS.

In contrast to the promoter region, the CpGs within repeat B1 displayed highly consistent paternal allele-specific methyl-
Methylation Sequencing Analysis of 5' H19 Regions
ation in samples with normal imprinting supporting the view that the 400-bp upstream repeats may harbor the H19 methylation imprint in humans (23). Our methylation analysis also refined 3′ and 5′ boundaries of the potential imprinting mark. The location of a 3′ boundary was indicated by biallelic methylation of the CpGs within repeat A1 at −2.0 kb relative to the H19 transcription start. The 5′ boundary of the methylation imprint was refined to between −4.4 and −5.6 kb, with HpaII site 25 being the most 5′ methylation site that was clearly differentially methylated. Since the 400-bp direct repeats extend to −5.3 kb, it seems conceivable that the methylation imprint might comprise the entire repeat region from −2.0 to −5.3 kb, but this remains to be proven.

A comparison of the results obtained for human H19 in the present investigation to those obtained for mouse upstream H19 regions (20, 31) reveals remarkable similarities in the distribution of methylation patterns. In both species the methylation imprint is located at approximately the same distance 5′ to the H19 transcription start and is characterized by uniform paternal allele-specific methylation. In contrast, the regions downstream of the imprint toward the H19 promoter seem to harbor non-clonal patterns of less pronounced differential methylation, and the region upstream of the imprint harbors biallelic methylation in both species. These analogies between mouse and human underline the importance of DNA methylation in this region in H19 imprinting.

The hypothesis that an epigenetic error at the H19 locus early in embryonal development may cause biallelic H19 silencing in Wilms tumor implies that the 400-bp direct repeats extend to −5.3 kb, it seems conceivable that the methylation imprint might comprise the entire repeat region from −2.0 to −5.3 kb, but this remains to be proven.

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