Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site

Gary A. Ulaner1, Thanh H. Vu1, Tao Li1, Ji-Fan Hu1, Xiao-Ming Yao1, Youwen Yang1, Richard Gorlick2, Paul Meyers2, John Healey3, Marc Ladanyi4 and Andrew R. Hoffman1,*

1Medical Service, VA Palo Alto Health Care System, and Department of Medicine, Stanford University, Palo Alto, CA, 94304, USA, 2Department of Pediatrics, 3Department of Orthopaedics and 4Department of Pathology, Memorial Sloan–Kettering Cancer Center, New York, NY 10021, USA

Received November 4, 2002; Revised December 3, 2002; Accepted December 11, 2002

The adjacent insulin-like growth factor 2 (IGF2) and H19 genes are imprinted in most normal human tissues, but imprinting is often lost in tumors. The mechanisms involved in maintenance of imprinting (MOI) and loss of imprinting (LOI) are unresolved. We show here that osteosarcoma (OS) tumors with IGF2/H19 MOI exhibit allele-specific differential methylation of a CTCF-binding site upstream of H19. LOI of IGF2 or H19 in OS occurs in a mutually exclusive manner, and occurs with monoallelic expression of the other gene. Bisulfite sequencing reveals IGF2 LOI occurs with biallelic CpG methylation of the CTCF-binding site, while H19 LOI occurs with biallelic hypomethylation of this site. Our data demonstrate that IGF2 LOI and H19 LOI are accompanied by reciprocal methylation changes at a critical CTCF-binding site. We propose a model in which incomplete gain or loss of methylation at this CTCF-binding site during tumorigenesis explains the complex and often conflicting expression patterns of IGF2 and H19 in tumors.

INTRODUCTION

Genomic imprinting is the preferential silencing of one parental allele due to epigenetic modifications. Insulin-like growth factor II (IGF2), which codes for a potent mitogen (1), and H19, a putative tumor suppressor (2), are two imprinted genes located adjacent to each other at chromosome 11p15.5 in humans. In most tissues IGF2 is maternally imprinted (3) and H19 is paternally imprinted (4). Loss of imprinting (LOI) in cancer, and may be involved in malignant transformation (5–7).

The mechanisms responsible for maintenance of imprinting (MOI) and loss of imprinting (LOI) are unresolved. One potential mechanism involves differentially methylated regions (DMRs) which act as epigenetic modifiers of allelic expression by recruiting proteins that specifically bind to methylated or unmethylated DNA. DMRs have been proposed to participate in the imprinting control of human IGF2 and H19. Within the IGF2 gene, Sullivan et al. (8) located a region spanning exons 2 and 3 which is differentially methylated, and is homologous to dmr 0 in the mouse insulin-like growth factor 2 (lgf2) gene. Loss of maternal-allele specific methylation in this region correlated with IGF2 LOI in Wilms tumors (8). Vu et al. (9) found no similar differentially methylated patterns around IGF2 exon 5 or 8.

The most notable DMR associated with IGF2 is in the region upstream of H19. There are four specific binding sites for the zinc finger protein CTCF (CCCTC-binding factor) in this region of mouse lgf2 (10–13). CTCF binds the unmethylated maternal allele and acts as an insulator between lgf2 and downstream enhancers, thereby suppressing lgf2 transcription. Methylation of the paternal allele prevents CTCF binding, thereby precluding the establishment of the insulator and allowing lgf2 transcription.

In humans, the DMR upstream of H19 contains seven CTCF-binding sites (11). This area is differentially methylated in most normal human tissues, with the paternal allele being methylated and the maternal allele being unmethylated (9,14,15). Only the sixth of the seven CTCF-binding sites has been demonstrated to have allele-specific differential methylation (16), and the loss of differential methylation at this site correlates with LOI in
Wilms tumors (17), bladder cancer (16), and colon cancer (18,19). Hypermethylation of the normally unmethylated allele was found in Wilms tumors with IGF2 LOI (17), while hypomethylation of the normally methylated allele was found in bladder cancers with H19 LOI (16). One group has found moderate hypermethylation in colon cancer with IGF2 LOI (18), although another claims hypomethylation is more prominent (19).

A paralogue of CTCF, known as BORIS (brother of the regulator of imprinted sites) has recently been cloned. CTCF and BORIS contain the same zinc finger domain, and thus may possess a similar DNA-binding potential (20). As the onset of BORIS expression correlates with genome-wide demethylation during male germ-line development, BORIS has been proposed to be involved in the removal of methylation marks (20). BORIS expression has been suggested in some tumors (21), but whether BORIS expression in tumors results in impaired DNA methylation is unknown.

Osteosarcoma (OS) is the most common primary bone tumor. We screened 72 osteosarcoma tumors from 62 patients for informative polymorphisms of IGF2 and H19, analyzed the expression of IGF2 and H19 alleles, and determined the methylation status of critical DMRs. Here we report IGF2/H19 LOI in OS. We show that BORIS is expressed in some OS tumors, and how BORIS expression may affect CpG methylation in this tumor. We demonstrate how CpG methylation of a critical CTCF-binding site may affect IGF2/H19 imprinting and we propose a model in which incomplete gain or loss of CpG methylation at this site explains the complex expression of IGF2 and H19 in tumors.

RESULTS

IGF2 and H19 genotypes in OS

A schematic of three IGF2 exon 9 polymorphisms and two H19 exon 5 polymorphisms evaluated in this study is shown in Figure 1. The genotypes at these polymorphisms were determined by PCR for 72 OS tumor samples from 62 patients, to identify samples that were informative for IGF2 and H19. Results of OS genotyping can be found in Table 1. Overall 28 patients were informative for IGF2 and 34 were informative for H19. Seventeen patients were informative for both IGF2 and H19. When more than one tumor sample was obtained from a single patient, these samples always displayed an identical genotype.

Allelic expression of IGF2 and H19 in OS

Samples that were heterozygous at one or more polymorphisms were analyzed for IGF2 and H19 allelic expression by reverse transcription PCR (RT–PCR). The results of allelic expression in OS can be found in Table 1. Overall, seven of 28 patients informative for IGF2 demonstrated loss of imprinting (LOI) of IGF2. Seven of 34 patients informative for H19 demonstrated LOI of H19. Of the 17 patients informative for both genes, IGF2 LOI and H19 LOI were mutually exclusive. No patient lost imprinting for both genes, even though eight patients with LOI for one gene were informative for the other (Table 2, IGF2 LOI patients nos 1, 3, 4 and 6 and H19 LOI patients nos 2, 3, 4 and 7). When more than one tumor sample was obtained from a single patient, these samples displayed identical allelic expression. Representative samples of OS genotypes and allelic expression are shown in Figure 2.

IGF2 promoter usage in OS

IGF2 may be transcribed from four alternate promoters (P1–P4), of which P3 and P4 are predominant in most tissues (22). While P2, P3, and P4 are maternally imprinted, P1 is normally biallelically expressed (23,24). Thus, biallelic expression of IGF2 may result from either LOI of promoters P2–P4, or from transcription from P1. To show that biallelic IGF2 expression in OS tumors was due to LOI, and not from P1 transcription, we performed multiplex PCR. Multiplex PCR was performed with 5′ primers specific for each of the four IGF2 promoters, and with a common labeled 3′ primer (Fig. 3A). Products from this multiplex PCR have been shown to reflect the relative abundance of the promoter-specific derived transcripts (25). Of 24 OS tumors examined, expression was predominantly from P3 and P4, and extremely low; if any, expression was seen from P1. Typical examples of IGF2 promoter usage from OS tumors are shown in Figure 3B.

Differentially methylated regions in OS

The methylation status of potential DMRs was determined by cloning and sequencing bisulfite treated DNA. The region upstream of H19 was examined with two pairs of PCR primers. The 5′-7962BT/H19-6413BT primer pair amplifies a region from 2374 to 1923 bp upstream of the H19 transcription start site, which contains the sixth of seven CTCF-binding sites upstream of H19 (Fig. 4A) (11). This region has been shown to be differentially methylated in normal human tissues, with one allele methylated and the other unmethylated (9). For most OS tumors, two single-nucleotide polymorphisms (SNPs) in this region allowed categorization of clones into groups based on allele of origin. In tumors with MOI of both IGF2 and H19, one allele was nearly entirely methylated in this region, while the other was nearly entirely unmethylated. As compared with samples with MOI, tumors with IGF2 LOI had striking methylation of the normally unmethylated allele, whereas tumors with H19 LOI had variable amounts of demethylation of the normally methylated allele. Demethylation in H19 LOI was most pronounced within the CTCF-binding site.

The H19-7574BT/H19-7959BT primer pair amplifies a region from 762 to 377 bp upstream of H19 near the H19 promoter (Fig. 4B). Two SNPs were also available in this region to categorize clones by allele of origin. Tumors with MOI or IGF2 LOI showed similar methylation patterns in this region to patterns found in the other H19 upstream region (MOI tumors contained one methylated and one unmethylated allele, while tumors with IGF2 LOI had methylation of both alleles). However, unlike the previous region, H19 LOI tumors had only one methylated and one unmethylated allele.

One previous report used restriction enzyme digestion and Southern blotting to describe differential methylation of a region spanning exons 2 and 3 of IGF2 in Wilms tumors (8).
We designed two sets PCR primers for bisulfite sequencing of this region (Fig. 4C). One SNP polymorphism was found to categorize cloned segments by allele of origin. Bisulfite sequencing revealed that methylation of this region was not uniform in OS. No predominantly methylated or unmethylated alleles could be seen.

We used MR–PCR (methylation restriction PCR), a simplified COBRA (combined bisulfite restriction analysis) method, to verify and expand the findings of bisulfite cloning and sequencing. In the H19 upstream region, the 452 bp PCR fragment produced by the H19-5962BT/H19-6413BT primer pair would contain BstUI sites (CGCG) if CpG dinucleotides were methylated before bisulfite treatment (Fig. 5A; these 3 CpG sites correspond to CpG 6190, 6192 and 6194 in Fig. 4A). MR–PCR shows that, at this CTCF-binding site, eight tumors with MOI contained both methylated and unmethylated CpG sequences. The majority of sequences in eight H19 LOI tumors were unmethylated. Restriction digestion was complete in eight IGF2 LOI tumors, representing completely methylated sequences. These results agree with bisulfite sequencing and expand the methylation analysis to a total of 24 tumors.

In the H19 promoter region, the 386 bp fragment produced by the H19-7574BT/H19-7959BT primer pair would contain a unique HpyCH4 IV site (ACGT) if the CpG at 7898 was originally methylated (Fig. 5B, compare with Fig. 4B). HpyCH4 IV digestion shows that tumors with MOI contained both methylated and unmethylated CpG dinucleotides at this site. Most H19 LOI tumors likewise contained both methylated and unmethylated CpG dinucleotides. IGF2 LOI tumors were entirely methylated. Of note, two H19 LOI tumors from the same patient (OS158 and OS178) produced restriction fragments, suggesting that they were entirely unmethylated at this site, and this corresponds with bisulfite sequencing of OS158.

In the IGF2 exon 2/3 region, the 342 bp fragment produced by the IGF2-65759BT/IGF2-65418BT primer pair would contain a unique Taq I site (TCGA) if the CpG at 65 608 was originally methylated.

**Figure 1.** IGF2 and H19 polymorphisms. (A) Schematic of the IGF2/H19 region of chromosome 11p15.5. Locations of PCR primers are indicated by numbered arrows. Asterisks represent location of 32P labeling. Three polymorphisms were analyzed in IGF2 Exon 9 (Apa I at bp 819, 19 bp deletion of bp 1180–1199, and 4 bp insertion at bp 1448). IGF2 bases are numbered as found in GenBank accession number X07868. Two polymorphisms were analyzed in H19 Exon 5 (Alu I at bp 10378 and Rsa I at bp 10736). H19 bases are numbered as found in GenBank accession number AF087017. Predicted products for the A and B alleles of each polymorphism are drawn below the schematic. (B) Actual products of genotype analysis. Representative samples of A homozygotes, B homozygotes, and A+B heterozygotes are shown for each polymorphism. The PCR primers for the H19 Alu I polymorphism cross an intron, and thus products from cDNA are smaller than those from genomic DNA.
methylated (Fig. 5C, compare with Fig. 4C). In contrast to the upstream H19 region and the H19 promoter region, in this region products representing both methylated and unmethylated CpG dinucleotides were seen in all tumors regardless of imprinting status. This confirms the heterogeneity of methylation seen in this region by bisulfite sequencing (in Fig. 4C).

**CTCF and BORIS expression in MOI and LOI**

Previously, CTCF expression has been found to be nearly ubiquitous while BORIS has been found only in the testis. In OS, CTCF was ubiquitously expressed (Fig. 6). BORIS expression was observed after RT–PCR in nine of 24 OS

### Table 1. Genotypes and allelic expression for OS tumors. Tumors are listed by their unique Memorial Sloan–Kettering sample numbers. In some cases, more than one sample was obtained from an individual patient. Multiple samples from the same patient were concordant. From top to bottom, seven patients with IGF2 LOI, seven patients with H19 LOI, nine patients informative for both IGF2 and H19 with MOI, eight patients informative only for IGF2 with MOI, and 14 patients informative only for H19 with MOI. Seventeen patients (19 tumors) were non-informative and are not listed to conserve space.

<table>
<thead>
<tr>
<th>Genotypes and allelic expression for OS tumors</th>
<th>Genotypes and allelic expression for OS tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loss of imprinting of IGF2</td>
<td>Loss of imprinting of IGF2</td>
</tr>
<tr>
<td>Genotype expressed</td>
<td>Genotype expressed</td>
</tr>
<tr>
<td>Loss of imprinting of H19</td>
<td>Loss of imprinting of H19</td>
</tr>
<tr>
<td>Genotype expressed</td>
<td>Genotype expressed</td>
</tr>
<tr>
<td>Maintenance on imprinting of both IGF2 and H19</td>
<td>Maintenance on imprinting of both IGF2 and H19</td>
</tr>
<tr>
<td>Genotype expressed</td>
<td>Genotype expressed</td>
</tr>
<tr>
<td>Maintenance on imprinting IGF2</td>
<td>Maintenance on imprinting IGF2</td>
</tr>
<tr>
<td>Genotype expressed</td>
<td>Genotype expressed</td>
</tr>
<tr>
<td>Maintenance on imprinting H19</td>
<td>Maintenance on imprinting H19</td>
</tr>
<tr>
<td>Genotype expressed</td>
<td>Genotype expressed</td>
</tr>
</tbody>
</table>

#### IGF2 polymorphisms
- **APAI site at bp819**
  - Genotype expressed: A/B a/b
- **19 bp delete at bp1180**
  - Genotype expressed: A/B a/b
- **4 bp insert at bp1448**
  - Genotype expressed: A/B a/b

#### H19 polymorphisms
- **Apa I site at bp10378**
  - Genotype expressed: A/B a/b
- **Rsa I site at bp10376**
  - Genotype expressed: A/B a/b
tumors, although expression was low, requiring 36 cycles of PCR to produce products comparable to those found after 30 cycles for CTCF. BORIS was found in six of eight tumors with \( H19 \) LOI, but was not found in tumors with \( IGF2 \) LOI. BORIS was expressed in three of eight tumors that maintained imprinting of both \( IGF2 \) and \( H19 \).

**DISCUSSION**

\( IGF2 \) and/or \( H19 \) LOI commonly occurs in cancers, and may be involved in malignant transformation (5–7). \( IGF2 \) LOI, leading to biallelic production of a potent growth factor, may provide augmented growth promoting signals to a tumor. \( IGF2 \) appears to be important for OS growth, as suppression of \( IGF2 \) in OS cell lines leads to growth inhibition (26). The mechanism by which \( H19 \) LOI may promote tumorigenesis is more difficult to explain, but \( H19 \) LOI may be a marker of more widespread epigenetic dysregulation.

Our understanding of the mechanisms underlying maintenance and loss of imprinting is incomplete. In the mouse, the \( H19 \) DMR is required for both \( H19 \) and \( Igf2 \) imprinting (27). A current model suggests that the maternal unmethylated \( H19 \) DMR allows CTFC binding, establishing an insulator between \( Igf2 \) and downstream enhancers, which prevents \( Igf2 \) transcription and supports \( H19 \) transcription. On the methylated paternal DMR, CTFC binding is prevented, allowing for \( Igf2 \) transcription, while \( H19 \) is silenced (10,11). This model would predict that if the DMRs of both alleles were methylated, then \( Igf2 \) would be biallelically expressed and \( H19 \) would be silenced. Likewise, this model would predict that if the DMRs of both alleles were unmethylated, then \( H19 \) would be biallelically expressed and \( Igf2 \) would be silenced. Indeed, this predicted reciprocal pattern of gene expression was observed both in normal human development (28) and in Wilms tumors (29–31), where \( IGF2 \) LOI was found with biallelic DMR methylation and suppression of \( H19 \) expression. In this study of osteosarcoma, \( IGF2 \) LOI and \( H19 \) LOI

Table 2. PCR primers used in this study. PCR primers are named for the gene they target and the position of the 5' base as documented in Genebank (accession number AF087017 for all \( H19 \) primers, X07868 for \( IGF2 \) exon 9 genotyping, AC006408 for \( IGF2 \) promoter usage analysis and \( IGF2 \) exon 2/3 cloning, NM006565 for CTFC, and AF336042 for BORIS). Primers are labeled BT when they were designed to anneal to DNA that has been bisulfite treated. Primers for \( IGF2 \) promoter usage analysis are complementary to AC006408 sequence. *#* is the primer number used to catalog primers in our laboratory. Optimal annealing temperatures were determined by PCR on a gradient thermal cycler.

<table>
<thead>
<tr>
<th>Primer</th>
<th>#</th>
<th>Sequence (5' to 3')</th>
<th>Optimal annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotyping and allele expression of ( IGF2 ) and ( H19 ) polymorphisms (Figs. 1 and 2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( IGF2-258 )</td>
<td>3039</td>
<td>CTT GGA CTT TGT T/GTA GTC AAA TCG GC</td>
<td>65</td>
</tr>
<tr>
<td>( IGF2-857 )</td>
<td>3072</td>
<td>GCG GTA CCA GCG AGG TGC CCA C</td>
<td>65</td>
</tr>
<tr>
<td>( IGF2-1093 )</td>
<td>2266</td>
<td>CTC ATA CTT TAT GCA TCC CCG CAG CTA C</td>
<td>65</td>
</tr>
<tr>
<td>( IGF2-1226 )</td>
<td>2568</td>
<td>CTG TGT GTG TGT GCT GTC GTT T</td>
<td>67</td>
</tr>
<tr>
<td>( IGF2-1240 )</td>
<td>2241</td>
<td>CAC AAA CGC ACA GCA CAC GCA CAC ACA TGC</td>
<td>67</td>
</tr>
<tr>
<td>( IGF2-1470 )</td>
<td>2586</td>
<td>TTT GTG TGT GTG TGT GTG TGT AG</td>
<td>67</td>
</tr>
<tr>
<td>( H19-10242 )</td>
<td>2839</td>
<td>CTT TAC AAC CTC TGC ACT ACC TGA C</td>
<td>67</td>
</tr>
<tr>
<td>( H19-10423 )</td>
<td>2838</td>
<td>GAT GGT TCT TTT GAT GTG GTT TTA AGT</td>
<td>67</td>
</tr>
<tr>
<td>( H19-10709 )</td>
<td>2530</td>
<td>GGA GTT GTG GAG ACG GCC TTG AGT</td>
<td>67</td>
</tr>
<tr>
<td>( H19-10181 )</td>
<td>2490</td>
<td>CCA GTC ACC CGG CCC AGA TGG AG</td>
<td>67</td>
</tr>
<tr>
<td><strong>IGF2 promoter usage analysis (Fig. 3)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( IGF2-64067 )</td>
<td>3490</td>
<td>CAG TCC TGA GGT GAG CTG CTG TGG C</td>
<td>65</td>
</tr>
<tr>
<td>( IGF2-57451 )</td>
<td>1871</td>
<td>ACC GGG CAT TGC CCC CAG TCT CC</td>
<td>63</td>
</tr>
<tr>
<td>( IGF2-54755 )</td>
<td>1872</td>
<td>CGT CGC ACA TTC GGC CCC CGG GAC T</td>
<td>63</td>
</tr>
<tr>
<td>( IGF2-52524 )</td>
<td>1873</td>
<td>TCC TCC TCC TCC TGC CCC AGG G</td>
<td>63</td>
</tr>
<tr>
<td>( IGF2-51846 )</td>
<td>1605</td>
<td>CAG CAA TGC AGC AGC AGG CGA AGG C</td>
<td>65</td>
</tr>
<tr>
<td><strong>Cloning and restriction enzyme analysis of bisulfite-treated DNA (Figs. 4 and 5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H19-5962BT )</td>
<td>134</td>
<td>TGT TGA TGG TGG GGA AGG TGG GA</td>
<td>61</td>
</tr>
<tr>
<td>( H19-6413BT )</td>
<td>130</td>
<td>AAA GGG GAA TGA TGG TGG CAC TAC AAG GAG C</td>
<td>63</td>
</tr>
<tr>
<td>( H19-7574BT )</td>
<td>98</td>
<td>CTC (A/G)CC AAT CTC CAC TCC TGC ACT CCC AAC C</td>
<td>63</td>
</tr>
<tr>
<td>( H19-7599BT )</td>
<td>99</td>
<td>TTT TGG GGA TTT(C/T) CTA GGA TAT AGA TGG TGC CAC TAC AAG GAG C</td>
<td>63</td>
</tr>
<tr>
<td>( IGF2-65599BT )</td>
<td>1303</td>
<td>AAT CCC TTC ATT TCT CCA ACC TCA AC</td>
<td>61</td>
</tr>
<tr>
<td>( IGF2-65418BT )</td>
<td>1302</td>
<td>GAA ATT AGG ATT TTG GTA GTT TTA AGT</td>
<td>61</td>
</tr>
<tr>
<td>( IGF2-65310BT )</td>
<td>1301</td>
<td>CTC TTT TAA AAC TAC ACA AAA CCA CT</td>
<td>61</td>
</tr>
<tr>
<td>( IGF2-64972BT )</td>
<td>1300</td>
<td>GGT GGT TAG TAG GTA TAG GGA GGT A</td>
<td>61</td>
</tr>
<tr>
<td><strong>Genomic DNA controls for restriction enzyme digestions (Fig. 5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( H19-5962 )</td>
<td>134</td>
<td>TGT TGA TGG TGG GGA AGG TGG GA</td>
<td>70</td>
</tr>
<tr>
<td>( H19-6413 )</td>
<td>130</td>
<td>ACC GGG GAA TGA TGG TGG CAC TAC AAG GAG C</td>
<td>70</td>
</tr>
<tr>
<td>( H19-7574 )</td>
<td>1306</td>
<td>CTC GCC AGT CTC CAC TCC ACT C</td>
<td>70</td>
</tr>
<tr>
<td>( H19-7599 )</td>
<td>1307</td>
<td>TCC TGG GGA CTC GCC TAC CAC TCA AC</td>
<td>70</td>
</tr>
<tr>
<td><strong>RT-PCR of CTFC and BORIS (Fig. 6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CTFC-14K7 )</td>
<td>1396</td>
<td>GAA CCC ATT CAG GGG AAA AGC</td>
<td>65</td>
</tr>
<tr>
<td>( CTFC-1462 )</td>
<td>1397</td>
<td>TCG CAA GTG GAC ACC CAA ATC</td>
<td>62</td>
</tr>
<tr>
<td>( BORIS-1019 )</td>
<td>1398</td>
<td>CAG GCC CTA CAA GTG TAA CGA CTG CAA</td>
<td>62</td>
</tr>
<tr>
<td>( BORIS-1289 )</td>
<td>1399</td>
<td>GCA TTA TGC AGG CTT CTC ACC TGA GTG</td>
<td>62</td>
</tr>
</tbody>
</table>
occurred in a mutually exclusive manner, however biallelic expression of \textit{IGF2} or \textit{H19} was still accompanied by monoallelic expression of the other gene.

We used bisulfite sequencing and MR-PCR to evaluate the role of potential DMRs on \textit{IGF2} and \textit{H19} imprinting. Our findings are consistent with previous reports that in tissues with \textit{IGF2}/\textit{H19} MOI, the paternal allele is methylated and the maternal allele is unmethylated at both the \textit{H19} promoter region and the sixth of seven CTCF-binding sites upstream of \textit{H19} (9,14,15). In contrast to tissues with MOI, in tumors with LOI of \textit{IGF2} or \textit{H19} we found a striking loss of allele-specific methylation in these regions. Both bisulfite sequencing and MR-PCR show that hypermethylation of both regions accompanies \textit{IGF2} LOI, while hypomethylation of the CTCF-binding site accompanies \textit{H19} LOI. Thus, the switching of the methylation status at a critical CTCF-binding site can potentially explain the mutually exclusive pattern of \textit{IGF2} or \textit{H19} LOI in OS. It is interesting that the methylation status of the \textit{H19} promoter does not always correlate with \textit{H19} expression, whereas the methylation status of the CTCF-binding site does.

As the onset of BORIS expression in spermatocytes correlates with genome-wide demethylation during male germ-line development, it has been hypothesized that BORIS may act to remove methylation marks (20). Aberrant expression of BORIS has been suggested in different tumors (21), but the effect of BORIS expression on DNA methylation in tumors is unknown. We show here that \textit{BORIS} is expressed in OS.  

\textbf{Figure 2.} Representative samples of MOI (sample no. 168), \textit{H19} LOI (sample no. 132), and \textit{IGF2} LOI (sample no. 181). The ‘DNA’ column shows the results of genotyping for the three \textit{IGF2} and two \textit{H19} polymorphisms studied. The ‘cDNA’ column shows the results of allelic expression analysis by RT-PCR. The ‘-RT’ column shows controls for allelic expression analysis lacking the reverse transcription step, to verify products are derived from cDNA and not genomic DNA. As the primers for the \textit{H19} Alu I polymorphism cross an intron, products from cDNA are smaller than those from genomic DNA, and a ‘-RT’ control was therefore not needed.
BORIS expression was found predominately among tumors with biallelic hypomethylation of the critical CTCF-binding site, consistent with the idea that BORIS may be involved in removal of methylation marks.

A model of how methylation switching may affect IGF2/H19 imprinting is described in Figure 7. We propose that both gain-of-methylation and loss-of-methylation can occur within one tumor type, such as OS. Gain-of-methylation, the methylation of the normally unmethylated maternal allele, accompanies IGF2 LOI. Loss-of-methylation, the demethylation of the normally methylated paternal allele, accompanies H19 LOI. If gain or loss of methylation is not complete, a 'leaky' CTCF-induced insulator can result, which explains the expression of both IGF2 and H19 from the same parental allele. Therefore, biallelic expression of IGF2 (IGF2 LOI) can occur with monoallelic expression of H19 (and vice versa), as seen in this study, as well as in cervical (32), ovarian (33), and head and neck (34) carcinomas. If gain or loss of methylation in this region is complete, then biallelic expression of one gene may occur with silencing of the other. This pattern of methylation and gene expression frequently occurs in Wilms tumors (29–31). Our model also allows for the occurrence of LOI of both IGF2 and H19, which has been observed in testicular germ cell tumors (35) and rarely in Wilms' tumors (29). If gain-of-methylation of the maternal allele occurs concurrently with loss-of-methylation of the paternal allele in a population of tumor cells, then both IGF2 and H19 would be expressed biallelically. Thus, the complex and often

![Figure 3. IGF2 promoter usage in OS. (A) Schematic of the nine IGF2 exons. Four alternate promoter sites are labeled P1-P4. Location of PCR primers are indicated by numbered arrows. Asterisks represent location of [32P] labeling. Predicted PCR products for transcripts produced from each of the four promoters are drawn below the schematic. (B) Actual products from multiplex PCR. A Hep3B cell line that expresses IGF2 from all four promoters was used to show typical PCR products for transcripts from each promoter. OS tumors predominately used P3 and P4.](image-url)
Figure 4. Methylation status of individual CpG dinucleotides as determined by bisulfite sequencing. Location of PCR primers used for cloning are indicated by numbered arrows on the IGF2/H19 diagrams. An enlarged diagram is given for each cloned segment. Circles represent the locations of CpG dinucleotides. The locations of polymorphisms, allowing for the comparison of alleles, are represented by rectangles. Base numbering was performed according to GenBank accession numbers AF087017 for H19 and AC006408 for IGF2. Below is the methylation status of each CpG in two tumors with MOI (samples 168 and 176), for four tumors with LOI H19 (samples 132, 140, 178 and 186), and two tumors with LOI IGF2 (samples 104 and 181). Black circles represent methylated CpGs, white circles represent non-methylated CpGs. (A) H19 upstream region from base 5962 to 6413. The CTCF-binding region is boxed. (B) H19 promoter region from base 7574 to 7959. These primers were designed to anneal to the ‘bottom strand’ of genomic DNA, so they are complementary to the sequence found in GenBank. (C) IGF2 Exon 2/3 region from base 65759 to 64972. Accession number AC006408 includes the complement of IGF2, so base pair numbering in this diagram declines from 5' to 3'.
conflicting expression patterns of IGF2 and H19 in tumors may be explained by methylation switching of a CTCF-binding region.

There are other proposed models for IGF2 LOI. In the mouse, a silencer element has been demonstrated within the Igf2 gene (36,37), and a tissue-specific Igf2 enhancer has been located 32 kb upstream of H19 (38). To date, neither of these elements has been observed in humans. In Wilms’ tumors, Sullivan et al. (8) presented data from Southern blotting that suggests another DMR exists within the exon 2/3 region of IGF2 (8). Our results from bisulfite sequencing and MR–PCR show no consistent methylation patterns in this region in OS. Does altered DMR methylation necessarily lead to disruption of imprinting in all tumors? This does not seem likely. Although all tumors in this study with alterations in H19 DMR allele-specific differential methylation resulted in LOI of IGF2 or H19, Cui et al. (17) have shown in Wilms tumors that biallelic DMR methylation occurs in tumors without losing IGF2 imprinting.

In summary, the mutually exclusive pattern of IGF2 LOI and H19 LOI in OS can potentially be explained by CpG methylation switching of a CTCF-binding site. Biallelic methylation of this critical CTCF-binding site upstream of H19 accompanies IGF2 LOI, while biallelic hypomethylation...

Figure 4 continued.
of this region accompanies H19 LOI. The demethylation associated with H19 LOI co-occurred with BORIS expression, expanding the putative role of BORIS as a remover of methylation marks in the male germline, to a similar role in cancer.

MATERIALS AND METHODS

Tumor samples and clinical data
Tumor samples were obtained from patients undergoing surgery at Memorial Sloan–Kettering Cancer Center after obtaining written informed consent under a protocol approved by The Memorial Hospital Institutional Review Board. Tissue obtained at the time of surgery was confirmed by pathologic examination and samples were flash frozen and stored at –80°C until used in molecular studies.

Nucleic acid collection from tissues
Genomic DNA was collected from tissues using a solution of 4 M guanidinium thiocyanate, 25 mM sodium citrate, 1% 2-mercaptoethanol, and 0.5% Sarkosyl as described previously (39). The homogenate was extracted with phenol/chloroform and then precipitated with 2-propanol. The DNA pellet was washed with ethanol and dissolved in distilled water. RNA was collected from tissue using Tri-reagent (Sigma, St Louis, MO, USA).

Genotyping of IGF2 and H19 polymorphisms
Genotypes of 72 OS tissues for three potential IGF2 polymorphisms and two potential H19 polymorphisms were determined by PCR of genomic DNA as diagrammed in Figure 1. Each PCR was performed in 6 μl volume under liquid wax. Reactions contained 200 ng of DNA, 0.1 μM appropriate
primers, 50 μM deoxynucleoside triphosphate, and 0.2 units Klen Taq I (Ab Peptides, St Louis, MO, USA). One primer from each pair was end-labeled with γ-[32P]ATP. PCR conditions were 95°C for 90 s, followed by 29 cycles of 95°C for 25 s and optimal annealing temperature for 1 min, and finally 72°C for 10 min. Optimal annealing temperatures for each primer pair was determined by PCR on a gradient thermal cycler, and are listed in Table 2.

Determination of three genotype polymorphisms required restriction enzyme digestion (Apa I, Alu I or Rsa I). For these samples, non-labeled primers were used until the last PCR cycle, when one [32P] labeled primer was added. Adding labeled primer during the last PCR cycle prevents formation of labeled heteroduplexes (40), and thus reduces errors in genotyping due to the failure of restriction enzymes to digest heteroduplexes. One microliter of PCR products was mixed with two units of appropriate enzyme (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 μl, and digested at 32°C (Apa I) or 37°C (Alu I and Rsa I) for 6 h.

Products were electrophoresed on 5% polyacrylamide-urea gel and visualized by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Expression of IGF2 and H19 alleles

Samples that were heterozygous at IGF2 and/or H19 polymorphisms were analyzed for allelic expression by RT–PCR. RNA samples were treated with DNase I (Invitrogen) in the presence of RNase inhibitor (Invitrogen) to eliminate contaminating genomic DNA. Total RNA (0.4 μg), random hexamer and M-MLV reverse transcriptase (Invitrogen) were used for cDNA production. cDNA samples were PCR-amplified with primers and conditions used for genotyping, but for only 27 cycles. For primer pairs that did not cross intron/exon boundaries, a control PCR was performed without a reverse-transcription step, to verify that products were derived from cDNA and not genomic DNA. Products were electrophoresed on 5% polyacrylamide–urea gel and visualized by a

Figure 5. Restriction enzyme analysis of individual CpG dinucleotides. The schematics on the left show the location of the CpG dinucleotides analyzed, and the predicted products based on whether the original CpG dinucleotides were methylated or unmethylated. On the gels, from left to right, are a base pair marker, a fully uncut control, a fully cut control, and products from eight MOI, eight LOI H19 and eight LOI IGF2 tumors. The controls were PCR amplified from non-bisulfite treated genomic DNA with primers designed to produce the same size products as the BT primers amplify from bisulfite treated DNA. Since these controls came from non-bisulfite treated DNA, their sequence is unchanged, and they are able to be fully restriction enzyme digested. (A) Analysis of CpGs at bases 6190–6195 in the CTCF-binding region upstream of H19. Of note, there is a second potential BstU I site (bases 6280–6283), which would result in a 330 bp-labeled product if the 6190–6195 region was unmethylated, while the 6280–6283 region was methylated. However, no product was seen representing this possibility. (B) Analysis of CpG at base 7898 upstream of H19. (C) Analysis of CpG at base 65 608 of IGF2. Since the Taq I site is created by bisulfite treatment (see schematic), there is no cut control on this gel.
were denatured in 20°C. DNA was bisulfite treated, ampliﬁed by PCR, cloned and sequenced. One microliter of bisulfite-treated DNA was PCR-ampliﬁed with appropriate primers in a total volume of 3 μl covered with liquid wax. One primer from each pair was end-labeled with γ-[32P]ATP (see Table 2). The four 5′ primers compete for the common 3′ primer. Products have been shown to reﬂect the relative abundance of the promoter-speciﬁc derived transcripts (25). PCR conditions were the same as for genotyping.

Bisulfite treatment of genomic DNA

Bisulfite treatment of genomic DNA efﬁciently converts unmethylated cytosines to uracil, while 5-methylcytosine remains unchanged (41). Two micrograms of genomic DNA were denatured in 20 μl 0.3 M NaOH for 20 min at 37°C, and then placed on ice. A 220 μl aliquot of 3.5 M sodium bisulfite containing 1 mM hydroquinone was added, and the solution was covered with liquid wax. The solution was incubated at 0°C for 12 h, then 50°C for 8 h. Resulting bisulfite treated DNA was puriﬁed using QIAEX II Extraction Kit (Qiagen, Valencia, CA, USA) and Centri-Spin20 Columns (Princeton Separations, Adelphia, NJ, USA). From 50 μl ﬁnal volume in water, 1 μl was used for each subsequent analysis.

Cloning and sequencing of bisulfite-treated DNA

To determine the methylation status of cytosines in genomic DNA, DNA was bisulfite-treated, ampliﬁed by PCR, cloned and sequenced. One microliter of bisulfite treated DNA was PCR-ampliﬁed with appropriate primers in a total volume of 3 μl covered with liquid wax. One primer from each pair was end-labeled with γ-[32P]ATP. PCR conditions were 95°C for 90 s, followed by 30 cycles of 95°C for 25 s, optimal annealing temperature for 45 s, and 70°C for 45 s, and ﬁnally 70°C for 10 min. PCR products were resolved on 5% acrylamide gels. Small pieces of gel containing the desired product were cut out and eluted in 100 μl water for 20 min at 90°C. One microliter of this elution was subjected to a second round of PCR with similar conditions except with non-labeled primers and only 16 cycles. One microliter samples of the second ampliﬁcation were resolved on 1% agarose gels with ethidium bromide to verify products were of the anticipated size. Successful PCR products were then cloned into the pCR2.1-TOPO vector (Invitrogen), transformed into TOP10 One Shot E.coli (Invitrogen), and plasmid DNA was collected by QIAprep Spin Mini-prep kit (Qiagen, Valencia, CA, USA). Automated sequencing of DNA was performed using Big-Dye (Perkin-Elmer, Wellesley, MA, USA).

Restriction enzyme analysis of bisulfite-treated DNA

The methylation status of individual cytosines can be assessed by bisulfite treatment of DNA, PCR and digestion with a restriction enzyme speciﬁc for the unchanged DNA sequence. These enzymes will only cut bisulfite-treated DNA if the cytosines being evaluated are methylated, and thus unchanged by bisulfite treatment. This method has been referred to as MR–PCR (42), and is a simpliﬁed version of COBRA (combined bisulfite restriction analysis) (43).

Bisulfite-treated DNA was PCR ampliﬁed for 30 cycles and then eluted as described in the cloning section above. One microliter of elution was ampliﬁed in a second round of PCR with similar conditions, but for only 16 cycles. Three microliters of products from this second round of PCR were digested with 10 units of appropriate restriction enzyme (NEBl, Beverly, MA, USA) in a total volume of 40 μl for 6 h (37°C for HpyCH4 IV, 60°C for BstU I, and 65°C for Tą l I). Products were electrophoresed on 5% polyacrylamide–urea gel and visualized by a PhosphorImager.

Fully cut and uncut controls were produced by PCR ampliﬁcation of non-bisulfite-treated DNA. Primers were designed to anneal to genomic DNA and amplify products of the same size as those produced from bisulfite-treated DNA with BT primers. PCR conditions were the same as for genotyping. Restriction enzyme digestion conditions were the same as above.

RT–PCR of CTCF and BORIS

cDNA samples were PCR ampliﬁed with gene speciﬁc primers (see Table 2), which crossed intron/exon boundaries in order to verify that PCR products were derived from cDNA and not from genomic DNA. PCR conditions for CTCF ampliﬁcation were 95°C for 90 s, followed by 30 cycles of 95°C for 25 s, 65°C for 45 s, and 72°C for 45 s, and ﬁnally 72°C for 10 min. PCR conditions for BORIS were 95°C for 90 s, followed by 36 cycles of 95°C for 25 s, 62°C for 45 s, and 72°C for 45 s, and ﬁnally 72°C for 10 min.

PhosphorImager. The threshold for scoring a sample as LOI was a ratio of less than 5:1 between the more abundant and less abundant alleles. In general, samples with LOI demonstrated a ratio of 1:1 to 2:1 between expressed alleles.

IGF2 promoter usage analysis

cDNA samples were PCR ampliﬁed with four promoter-speciﬁc 5′ primers and a common 3′ primer end-labeled with γ-32P ATP (see Table 2). The four 5′ primers compete for the common 3′ primer. Products have been shown to reﬂect the relative abundance of the promoter-speciﬁc derived transcripts (25). PCR conditions were the same as for genotyping.

Restriction enzyme analysis of bisulfite-treated DNA

The methylation status of individual cytosines can be assessed by bisulfite treatment of DNA, PCR and digestion with a restriction enzyme speciﬁc for the unchanged DNA sequence. These enzymes will only cut bisulfite-treated DNA if the cytosines being evaluated are methylated, and thus unchanged by bisulfite treatment. This method has been referred to as MR–PCR (42), and is a simpliﬁed version of COBRA (combined bisulfite restriction analysis) (43).

Bisulfite-treated DNA was PCR ampliﬁed for 30 cycles and then eluted as described in the cloning section above. One microliter of elution was ampliﬁed in a second round of PCR with similar conditions, but for only 16 cycles. Three microliters of products from this second round of PCR were digested with 10 units of appropriate restriction enzyme (NEBl, Beverly, MA, USA) in a total volume of 40 μl for 6 h (37°C for HpyCH4 IV, 60°C for BstU I, and 65°C for Tą l I). Products were electrophoresed on 5% polyacrylamide–urea gel and visualized by a PhosphorImager.

Fully cut and uncut controls were produced by PCR ampliﬁcation of non-bisulfite-treated DNA. Primers were designed to anneal to genomic DNA and amplify products of the same size as those produced from bisulfite-treated DNA with BT primers. PCR conditions were the same as for genotyping. Restriction enzyme digestion conditions were the same as above.

RT–PCR of CTCF and BORIS

cDNA samples were PCR ampliﬁed with gene speciﬁc primers (see Table 2), which crossed intron/exon boundaries in order to verify that PCR products were derived from cDNA and not from genomic DNA. PCR conditions for CTCF ampliﬁcation were 95°C for 90 s, followed by 30 cycles of 95°C for 25 s, 65°C for 45 s, and 72°C for 45 s, and ﬁnally 72°C for 10 min. PCR conditions for BORIS were 95°C for 90 s, followed by 36 cycles of 95°C for 25 s, 62°C for 45 s, and 72°C for 45 s, and ﬁnally 72°C for 10 min.
Figure 7. Model of methylation switching of a CTCF-binding site and IGF2/H19 imprinting in OS. Triangles represent CTCF-binding site no. 6 and squares represent the H19 promoter region. The DMR upstream of H19 contains both of these elements. Shading of triangles or squares represents light, heavy or full CpG methylation of these elements. Partial (light or heavy) methylation of the CTCF-binding site creates ‘leaky’ insulators, which are depicted by increasing space between CTCF and its binding site. Expression from the maternal (red) or paternal (blue) allele is represented as colored arrows. E = enhancer region. M = methyl groups on DNA preventing CTCF binding. In MOI tumors: as is the case with normal tissues (10,11), the maternal DMR is unmethylated, allowing CTCF binding and the establishment of an insulator which promotes H19 transcription. The paternal DMR is methylated, preventing insulator function and promoting IGF2 transcription. In LOI IGF2 tumors: there is a gain-of-methylation of the normally unmethylated maternal DMR, establishing a ‘leaky’ insulator, and allowing both IGF2 and H19 to be expressed from the maternal allele. In LOI H19 tumors: there is a loss-of-methylation of the normally methylated paternal sixth CTCF-binding site, establishing a ‘leaky’ insulator, and allowing both IGF2 and H19 to be expressed from the paternal allele. An interesting possibility is the role of BORIS in the removal of methylation marks from the paternal CTCF-binding site, which accompanies H19 LOI.
ACKNOWLEDGEMENTS

This study was supported by NIH Grant DK36054, NIH Institutional Training Grant DK072177, and the Medical Research Service of the Department of Veterans Affairs.

REFERENCES


