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Advances in Brief

Loss of Imprinting in Colorectal Cancer Linked to Hypomethylation of *H19* and *IGF2*[1]

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Abstract

Epigenetic alterations in human cancers include global DNA hypomethylation, gene hypomethylation and promoter hypermethylation, and loss of imprinting (LOI) of the insulin-like growth factor-II gene (*IGF2*). A mechanism for LOI described previously is hypermethylation of a differentially methylated region (DMR) upstream of the *H19* gene, allowing activation of the normally silent maternal allele of *IGF2*. Here we show that this mechanism does not apply to colorectal cancers, which show hypomethylation of the *H19* DMR as well as a DMR upstream of exon 3 of *IGF2*. This hypomethylation is found in both colorectal cancers and normal mucosa from the same patients, and in cell lines with somatic cell knockout of DNA methyltransferases *DNMT1* and *DNMT3B*. These data suggest that hypomethylation is a mechanism for LOI, that the popular *IGF2-H19* enhancer competition model for *IGF2* imprinting does not apply to the human colon, and that an alternative model for LOI would involve a transcriptional repressor acting on the normally silent maternal allele of *IGF2*.

Introduction

Epigenetic alterations in human cancer, *i.e.*, alterations in the genome other than the DNA sequence itself, were first described in 1983 by Feinberg and Vogelstein (1), who found widespread hypomethylation of genes in CRCs[3] and in premalignant adenomas. Epigenetic abnormalities identified subsequently include global genomic hypomethylation (2), promoter hypermethylation of CpG islands (3, 4), and LOI (5, 6), or loss of the normal parent of origin-dependent gene silencing, affecting at least the genes *IGF2*, *PEG1*, *p73*, and *LIT1* (5–11). LOI of *IGF2* causes overexpression of *IGF2* (12), an important autocrine growth factor in cancer. LOI was first identified in embryonal tumors in childhood, including Wilms' tumor, in which it is the most common molecular alteration (5, 6), as well as rhabdomyosarcoma (13) and hepatoblastoma (14). LOI was also later found in common adult malignancies including ovarian (15), colon (16), lung (17), and bladder cancer (18), as well as chronic myelogenous leukemia (19). In CRC, LOI is particularly important because it is found commonly in both the tumor and normal tissue of patients with CRC, at ~3-fold higher frequency then in patients without colon tumors (16), and, thus, LOI may represent the only common alteration linked to cancer that is found in normal tissue.

In Wilms' tumors, approximately half of tumors appear to arise by an epigenetic mechanism involving LOI rather than genetic alterations involving, for example, *WT1* mutations and LOH, and the tumors with LOI appear in children who develop cancer at a later age, accounting for the bimodal age distribution of Wilms' tumor (12). LOI was linked to increased methylation, because Wilms' tumors with LOI of *IGF2*, *i.e.*, activation of the normally silent maternal allele, show aberrant methylation of the normally unmethylated maternal allele of a DMR upstream of the *H19* gene on the same chromosome (20, 21). This result is consistent with the enhancer competition model for regulation of *H19* imprinting. By this model, *IGF2* and *H19* promoters compete on the same chromosome for a shared enhancer, and access of the maternal *IGF2* allele to this enhancer is blocked by the *H19* DMR when unmethylated, likely because of the insulator activity of CTCF binding to the unmethylated *H19* DMR (Refs. 22–26). Indeed, we observed that in Wilms' tumor, methylation of the maternal *H19* DMR includes CTCF-binding sites (27). These results would suggest that increased or ectopic activity of a DNA methyltransferase might lead to aberrant methylation of the maternal *H19* DMR.

Therefore, we were surprised to observe that HCT116, a CRC line with normal imprinting of *IGF2*, is hypermethylated at *H19* and retains normal imprinting after somatic cell knockout of the maintenance DNA methyltransferase *DNMT1* but loses imprinting after subsequent somatic cell knockout of *DNMT3B* (28), a *de novo* methyltransferase, *i.e.*, that is able to methylate unmethylated sequences and is necessary for normal imprinting (29, 30). This result implies that the loss of methylation, rather than the gain of methylation, causes LOI in CRC. To better determine whether LOI in CRC involves hypomethylation or hypermethylation, we performed genomic sequencing analysis. Our results differ from past studies, and they also suggest a model of *IGF2* imprinting in at least the colon that differs from the conventional view of enhancer competition between *IGF2* and *H19*.

Materials and Methods

Bisulfite Sequencing Analysis. *H19* CTCF binding site 1 (CBS1) was analyzed as described earlier (27); CBS6 corresponds to GenBank nucleotides 7855–8192 (accession no. AF125183) and was analyzed after bisulfite treatment using primers 5′-GAGTTTGGGGGTTTTTGTATAGTAT-3′ and 5′-CTTAAATCCCAAACCATAACACTA-3′, followed by 5′-GTATATGGGTATTTTTTGGAGGT-3′ and 5′-CCATAACACTAAAACCCTCAA-3′, both annealing at 55°C. The *IGF2* DMR sequence analyzed corresponds to GenBank nucleotides 631–859 (accession no. Y13633), and was analyzed after bisulfite treatment using primers 5′-GGGAATGTTTATTTATGTATGAAG-3′ and 5′-TAAAAACCTCCTCCACCTCC-3′, annealing at 55°C, followed by 5′-TAATTTATTTAGGGTGGTGTT-3′ and 5′-TCCAAACACCCCCACCTTAA-3′, annealing at 50°C. Other conditions are as described earlier (27).

Methyltransferase Activity Analysis. *In vitro* functional analysis was performed using the 293/EBNA1 cell line as described (31) and the pcDNA3Myc vector containing full-length DNMT3B coding sequences, and p220.2 (32) as the assay plasmid. Cotransfected target DNA was digested with the methylation-sensitive restriction endonuclease *Hpa*II, and Southern blot was performed using p220.2 as a probe. All of the transfections were done in duplicate or triplicate for each experiment.

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[3] The abbreviations used are: CRC, colorectal cancer; LOI, loss of imprinting; LOH, loss of heterozygosity; DMR, differentially methylated region.

Analysis of *DNTM3B* Sequence and *IGF2* Imprinting. Direct PCR sequencing of genomic DNA was performed to analyze the sequence of *DNMT3B*. All of the coding exons published including exon-intron junctions were thoroughly examined. LOI of *IGF2* was assessed according to hot-stop PCR (33).

Results

Hypomethylation of *H19* and *IGF2* DMRs in DNA Methyltransferase Knockout Cell Lines. HCT116 cells show normal imprinting but undergo LOI of *IGF2* after somatic cell knockout of both *DNMT1* and *DNMT3B* (28), suggesting that the loss of methylation rather than the gain of methylation is responsible for LOI in these cells. To test this hypothesis, we examined directly the methylation of two DMRs that distinguish parental alleles in human cells: the *H19* DMR 5-kb upstream of *H19* and methylated on the paternal allele (*H19* active, *IGF2* silent); and the *IGF2* DMR within intron 2 of *IGF2* and methylated on the maternal allele (Ref. 34). Note that the DMRs in humans differ from the mouse, in which there are three rather than one DMR within *IGF2* (35). Bisulfite sequencing analysis of HCT116 cells, and HCT116 cells lacking *DNMT1*, *DNMT3B*, or both, revealed that in the double-knockout cells, which showed LOI, both the *H19* and *IGF2* DMRs, were extensively hypomethylated (Fig. 1). This hypomethylation was found in three separate double-knockout lines with LOI and in none of single-knockout or wild-type lines with normal imprinting (Table 1).

Table 1 *IGF2 imprinting status and methylation alterations in DNMT knockout cell lines*

Cell lines	*IGF2* LOI	Methylation status	
		H19 CBS1	*IGF2* DMR
WT[a]	No	Hyper	Half
T1KO-1	No	Hyper	Half
T1KO-2	No	Hyper	Half
3BKO-1	No	Hyper	Half
3BKO-2	No	Hyper	Half
DKO-1	Yes	Hypo	Hypo
DKO-2	Yes	Hypo	Hypo
DKO-3	Yes	Hypo	Hypo

[a] WT, HCT116 wild-type; T1KO, *DNMT1* knockout; 3BKO, *DNMT3B* knockout; DKO, double-knockout; Half, normal half-methylation; Hypo, hypomethylation; Hyper, hypermethylation.

Hypomethylation of *H19* and *IGF2* DMRs in Primary CRCs. To determine whether hypomethylation was also linked to LOI in primary colon cancers, we then analyzed 20 CRC informative for imprinting status of *IGF2* (heterozygous for a transcribed polymorphism) by reverse transcription-PCR, 12 with LOI and 8 with normal imprinting. All 8 of the CRC with normal imprinting showed the normal half-methylation pattern at the *IGF2* DMR, and all 12 of the CRC with LOI showed marked hypomethylation of the *IGF2* DMR ($P = 0.000007$; Figs. 2 and 3). In tumors with normal imprinting, the fraction of CpG sites that were methylated was 43.6 ± 10.9%, whereas in tumors with LOI the fraction of sites methylated was 10.9 ± 9.4% ($P < 0.0001$). In addition, for each DMR, 15–20 clones were independently sequenced from the PCR product of each bisulfite-treated sample, and each experiment was repeated at least once. We also observed hypomethylation of the *H19* DMR in CRC, although the differences were not absolute as in the case of the *IGF2* DMR, but were in marked contrast to Wilms' tumors with LOI (Table 2). These results also differ markedly from those of Nakagawa *et al.* (35), who reported hypermethylation of CBS6 in colorectal cancer. Finally, because LOI is found at increased frequency in both tumor and normal tissue of patients with CRC, we also examined the matched normal mucosa of 3 CRC patients whose tumors showed LOI. As we reported earlier (16), the matched normal mucosa also showed LOI of *IGF2*, although methylation had not been examined in that study. We found the same pattern of hypomethylation in the normal colonic mucosa in each patient as we found in tumors (Table 2), indicating that this epigenetic abnormality was not limited to the cancers.

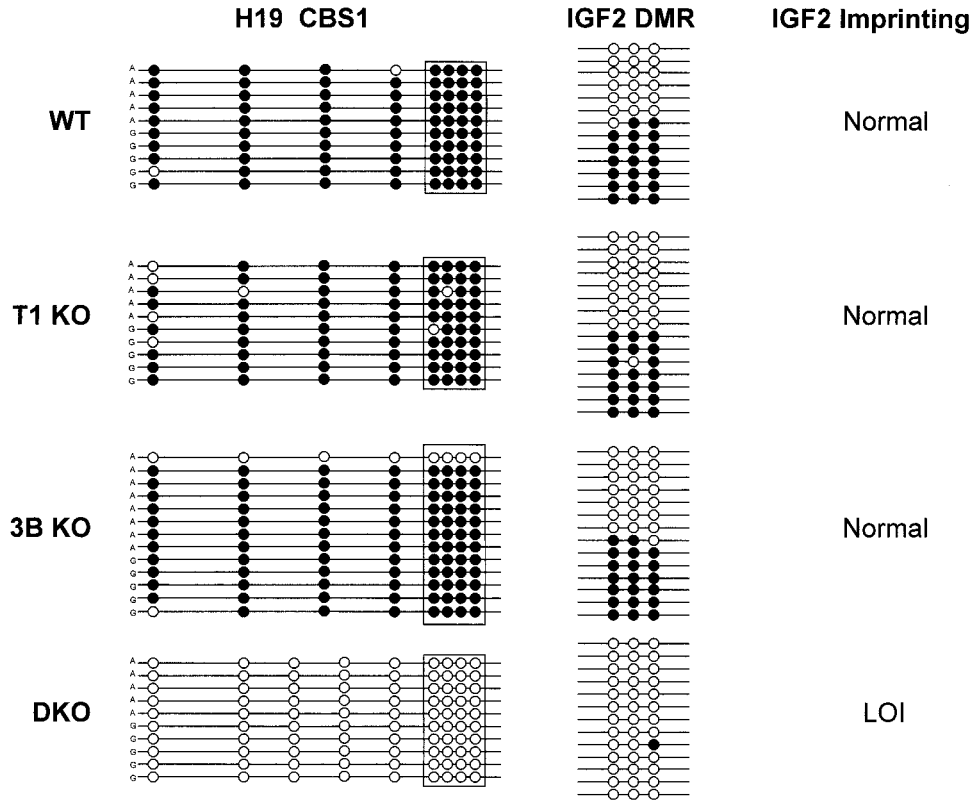

Fig. 1. Hypomethylation of *H19* and *IGF2* DMRs in CRC cell lines with LOI of *IGF2* and somatic cell knockout of DNA methyltransferase. HCT116 cells (*WT*), *DNMT1* knockout (*T1KO*), *DNMT3B* knockout (*3BKO*), and *DNMT1/DNMT3B* double-knockout (*DKO*) cells were analyzed by bisulfite genomic sequencing at the *H19* (CTCF binding site 1) and *IGF2* DMRs. By the enhancer competition model, biallelic methylation of the *H19* DMR should cause LOI of *IGF2*, but it does not in these cells. Rather, the *IGF2* DMR shows normal half methylation with normal imprinting and hypomethylation with LOI of *IGF2*.

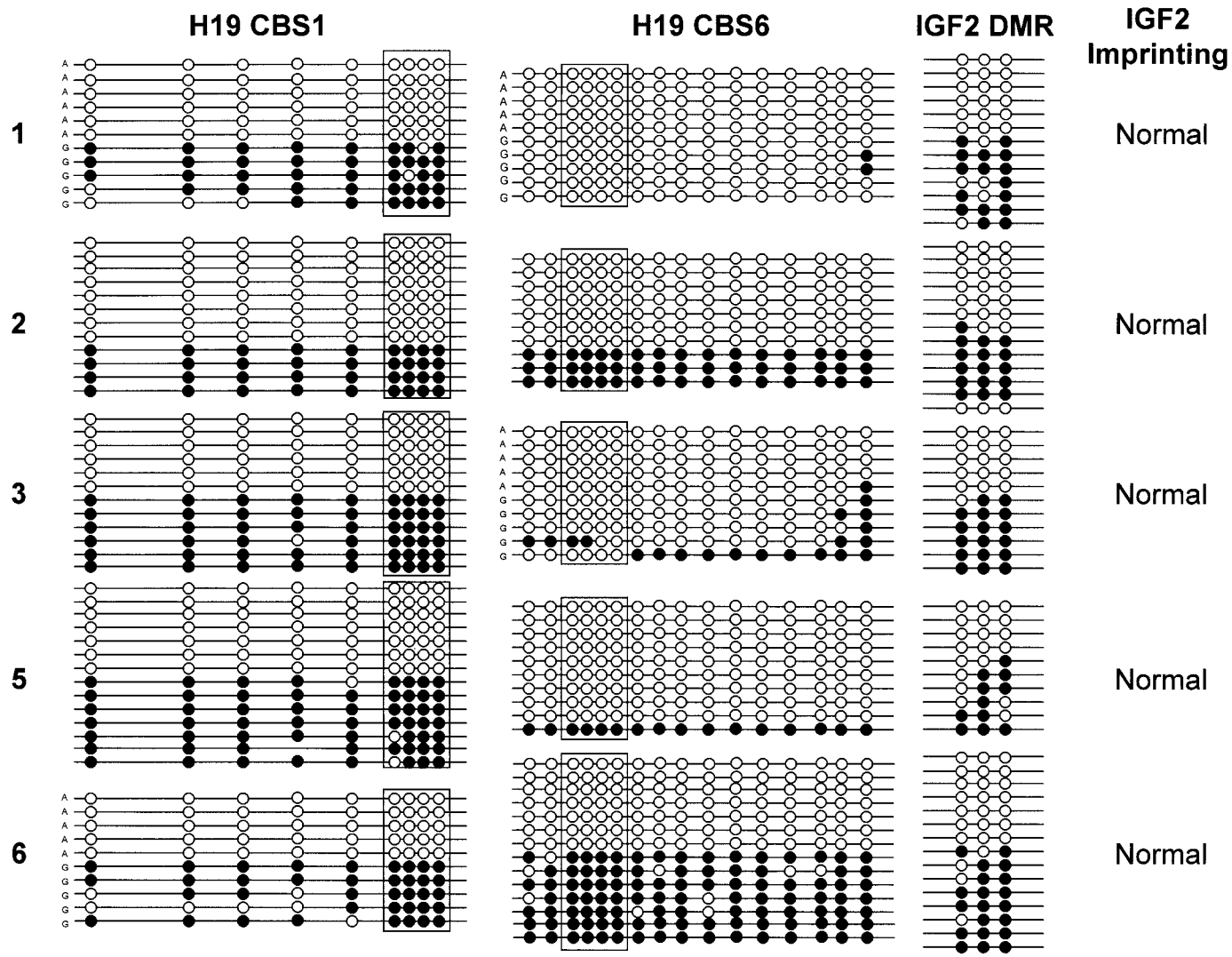

Fig. 2. Normal methylation of *IGF2* and variable methylation of *H19* DMRs in sporadic CRCs with normal imprinting of *IGF2*. The *H19* DMR shows variable hypomethylation unrelated to imprinting status of *IGF2*, whereas the *IGF2* DMR shows complete concordance of normal methylation and normal imprinting. Genomic DNA was treated with sodium bisulfite, and then was PCR amplified and subcloned before sequencing. Ten to 15 clones were sequenced for each sample. Each *line* represents a separate clone. (●), methylated CpG sites; (○), unmethylated CpG sites. Case number is shown on the left. Single nucleotide polymorphisms are shown on the left, distinguishing alleles.

Neutral Polymorphisms of *DNMT3B* in Human CRCs. Because LOI and hypomethylation were present in normal tissue, and DNMT3B appeared to play a role in LOI in HCT116 cells, we examined all 20 of the CRC for germ-line mutations in the DNMT3B gene. Six of 20 patients showed a single variation in the coding sequence leading to amino acid substitutions: G892T (G210W), G1390A (A376T), A1451G (Y396C), G2044A (V594I), G2086A (V608M), and T1436C (L391P). To distinguish between neutral and functional variants, we performed site-directed mutagenesis and transfection into 293/EBNA1 cells, together with an episomal vector, which was the target for *de novo* methylation. None of the variants disrupted *DNMT3B* methyltransferase activity (data not shown). Thus, these sequence variations represent neutral polymorphisms.

Discussion

This study has two major results. First, we report that hypomethylation, rather than hypermethylation, is linked to LOI of IGF2 in human CRC based on two lines of evidence. In CRC lines in which hypomethylation is induced artificially by *DNMT1/DNMT3B* double knockout, LOI is found only in the hypomethylated lines. Indeed, unmodified HCT116 cells with hypermethylation of the *H19* DMR exhibit normal imprinting, even though Wilms' tumors with hypermethylation of the same sites show LOI (27). Furthermore, we find that in primary human CRC, as well, LOI is linked to hypomethylation rather than hypermethylation. The latter result is in contrast to the findings of Nakagawa *et al.* (35), who reported hypermethylation of the *H19* in CRC with LOI of *IGF2*. It should be remembered that the first epigenetic alterations found in human cancer was hypomethylation of DNA (1) and that CRC show global hypomethylation even in the presence of specific sites of increased DNA methylation (2). Furthermore, the assumption that CpG islands are universally hypomethylated is incorrect, as imprinted genes show normal methylation, and we have also identified recently many normally methylated CpG islands in normal cells (36). Therefore, a more correct and inclusive view is that cancers show epigenetic instability, including global hypomethylation, and sites of both aberrantly increased and decreased methylation, that lead to altered gene regulation.

The second major result of this study is that normal imprinting in the colon and LOI in CRC is specifically linked to the methylation status of a DMR within *IGF2* and not *H19*. Thus, all 8 of the cancers with normal imprinting showed normal half-methylation of the *IGF2* DMR and all 11 of the cancers showed hypomethylation of this DMR,

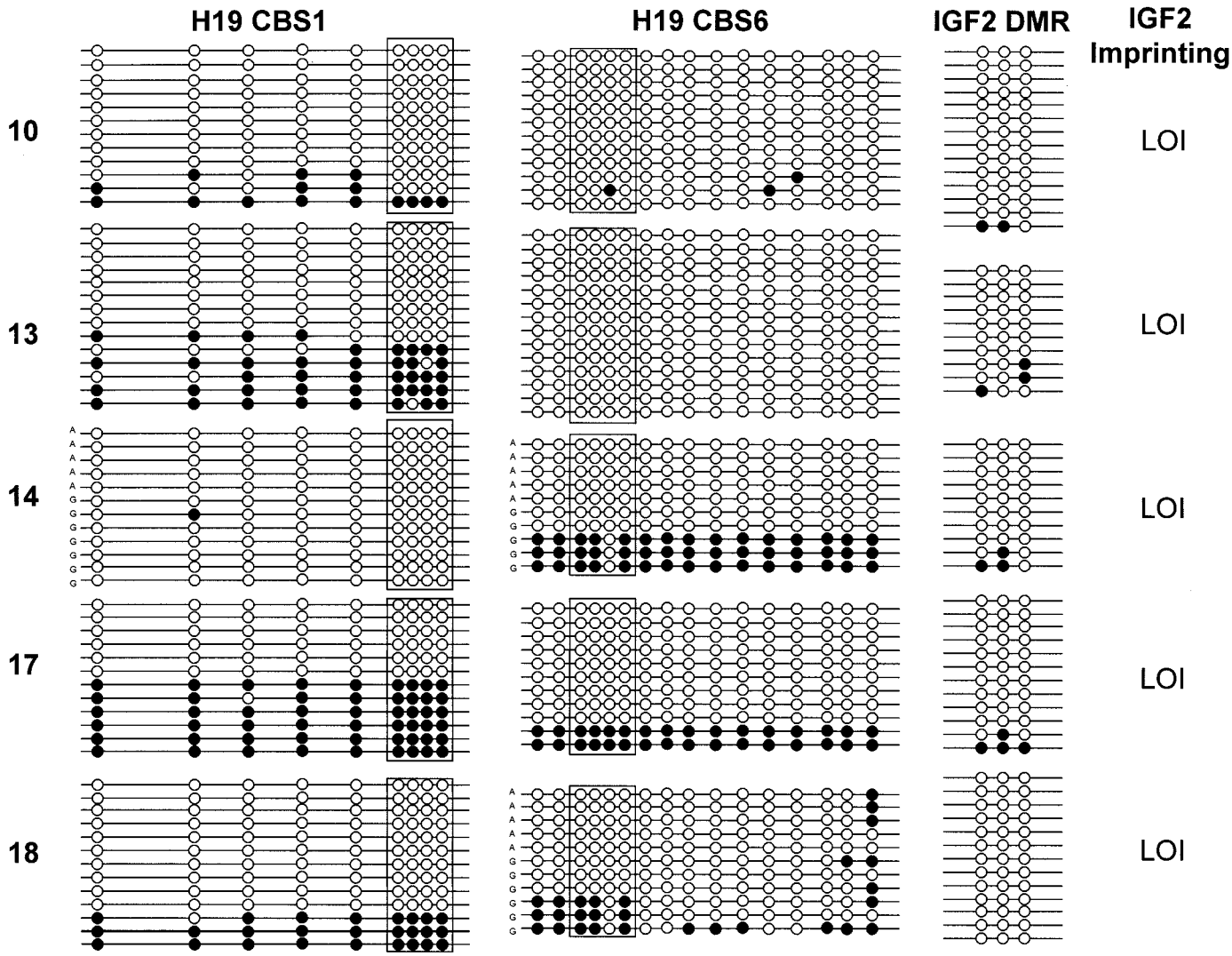

Fig. 3. Hypomethylation of *H19* and *IGF2* DMRs in sporadic CRCs with LOI. The *H19* DMR shows variable hypomethylation unrelated to imprinting status of *IGF2*, whereas the *IGF2* DMR shows complete concordance of hypomethylation and LOI. Labeling is as described in the legend to Fig. 2.

as well as 3 matched normal mucosal specimens that also showed LOI. Takai *et al.* (37) recently described partial or complete hypomethylation of the *H19* ICR in two of four bladder cancers, but no relationship to *H19* imprinting; *IGF2* was not examined in that study. We did not find any alteration of *H19* imprinting in the CRC examined here. We would argue that it is the *IGF2* DMR, not the *H19* DMR, that is important in maintaining imprinting in CRC. We had reported earlier that cancers with LOI also show LOI in the matched normal mucosa (16), so we would expect that this methylation abnormality is generally present in the colon of these cancer patients.

An important implication of this result is that it suggests a mechanism for regulation of *IGF2* imprinting independent of enhancer competition. By the enhancer competition model, *IGF2* and *H19* promoters compete on the same chromosome for a shared enhancer, and access of the maternal *IGF2* allele to this enhancer is blocked by the *H19* DMR when unmethylated, likely because of the insulator activity of CTCF binding to the unmethylated *H19* DMR (22–26). However, in CRC with LOI, the *H19* DMR is hypomethylated on both alleles, and hypomethylation of the *IGF2* DMR is specifically linked to LOI of *IGF2* in both primary CRC and in HCT116 cells in which methyltransferases have been disrupted experimentally.

Some clues to function are available from mouse studies, although

Table 2 *IGF2 imprinting status and methylation alterations in primary CRC*[a]

		Methylation status		
Sample no.	*IGF2* LOI	*H19* CBS1	*H19* CBS6	*IGF2* DMR
1T	No	Half	Hypo	Half
2T	No	Half	Hypo	Half
3T	No	Half	Hypo	Half
4T	No	Half	Half	Half
5T	No	Half	Hypo	Half
6T	No	Half	Half	Half
7T	No	Half	Half	Half
8T	No	Hyper	Half	Half
9T	Yes	Hypo	Hypo	Hypo
10T	Yes	Hypo	Hypo	Hypo
11T	Yes	Hypo	Hypo	Hypo
12T	Yes	Hypo	Hypo	Hypo
12N	Yes	Half	Hypo	Hypo
13T	Yes	Half	Hypo	Hypo
14T	Yes	Hypo	Hypo	Hypo
15T	Yes	Half	Hypo	Hypo
16T	Yes	Half	Half	Hypo
17T	Yes	Half	Hypo	Hypo
17N	Yes	Half	Hypo	Hypo
18T	Yes	Hypo	Hypo	Hypo
18N	Yes	Hypo	Hypo	Hypo
19T	Yes	Half	Half	Hypo
19N	Yes	Half	Half	Hypo
Fetus	No	Half	Half	Half

[a] Annotation as in Table 1.

it is difficult to relate mouse experiments precisely to the human, as the DMR sequences themselves differ between species. Nevertheless, the region corresponding to the human DMR studied here is in same physical relationship to human *IGF2* exons 2 and 3, as is mouse "DMR0" to mouse *Igf2* pseudoexons 1 and 2 (34). To date, no mouse knockout of DMR0 by itself has been reported, although deletion of DMR1, or of DMR0 and DMR1 together, lead to activation of the normally silent maternal allele of *Igf2* (38, 39). The mouse knockout experiments suggest the existence of a transcriptional repressor within *Igf2* (38, 39). We would agree with that hypothesis and additionally state that our results suggest that methylation of this human IGF2 DMR recruits transcriptional repressors to the maternal allele. By this model, hypomethylation would lead to LOI by loss of association of these repressors to the *IGF2* DMR. Our results also suggest two potentially valuable lines of experimentation: knockout of DMR0 in mouse and biochemical studies aimed at identifying factors of which the binding to the human *IGF2* DMR is lost in tumors with LOI.

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