Loss of imprinting of the insulin-like growth factor II gene occurs by biallelic methylation in a core region of H19-associated CTCF-binding sites in colorectal cancer

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We hypothesize that loss of imprinting (LOI) of the insulin-like growth factor II (IGF2) gene is associated with a predisposition to sporadic colorectal cancer. We confirmed a previously known strong correlation between LOI and microsatellite instability and showed that LOI was not a consequence of microsatellite instability or mismatch repair deficiency. LOI of IGF2 correlated strongly with biallelic hypermethylation of a core of five CpG sites in the insulator region of IGF2/H19, which is a known CTCF-binding element. As this methylation-dependent LOI was present in both tumors and normal colonic mucosa, it is possible that hypermethylation creates a field defect predisposing to cancer.

A methylator phenotype has been postulated to account for a subset of colorectal cancers (CRCs). In these sporadic, mostly right-sided tumors, hypermethylation of the CpG islands in the MLH1 promoter is associated with the absence of MLH1 protein, defective mismatch repair, and widespread microsatellite instability (MSI) (1–5). Among all CRCs the MSI-positive tumors account for some 10–15% (6, 7). Hereditary nonpolyposis colorectal cancer (HNPPC), caused by inherited germline mutations in MLH1 and MSH2, accounts for some 3% (6, 7). Among the manifestations of the methylator phenotype, promoter methylation frequently down-regulates other genes, notably p16 (8), and many other CpG islands methylated in colon cancer are also methylated in normal colonic mucosa (8). This generalized methylation increases with age (8) and may or may not be related to cancer. Globally, CpG island methylation shows a high degree of nonrandomness in distribution and tumor specificity (9).

Recently the growth-regulating insulin-like growth factor II (IGF2) gene was implicated in CRCs belonging to the MSI/methylator phenotype. Loss of imprinting (LOI) of IGF2 was found in 10 of 11 cancers with MSI but only 2 of 16 cancers without MSI (10). That LOI might characterize a cancer-prone state could be suggested based on the fact that it occurred in the unaffected colonic mucosa of most of the patients whose tumors showed LOI and in a small number of individuals without cancer (10). These findings raise the important question of whether LOI is also a manifestation of the methylator phenotype or an independent event. Genomic imprinting is defined as an epigenetic change leading to differential expression of the two parental alleles in somatic cells and usually involves allele-specific methylation of certain regions. IGF2 is one of many imprinted genes where only its paternal allele is expressed and the maternal allele is silent (11, 12). LOI is said to occur when the normally silenced allele is activated. LOI of IGF2 is observed frequently in a variety of childhood and adult tumors (13–16) and has been implicated in Beckwith–Wiedemann syndrome, a congenital overgrowth disorder that predisposes to embryonal tumors (17). The occurrence of LOI theoretically doubles the active gene dosage (18) and might contribute to tumor growth through its autocrine or endocrine effects. Recent studies in knock-out mice demonstrated that the supply of IGF2 could modify the growth of intestinal adenomas (19), thus implicating it in colorectal tumor progression and evolution.

In this study we addressed the question of whether LOI of IGF2 is a consequence of deficient mismatch repair and MSI, and whether it is associated with allele-specific methylation in the IGF2 region. To provide an easy and reproducible quantitative allele-specific assay of imprinting, we developed and validated a simple primer extension assay. Our results support the concept that sporadic CRC with MSI is associated with hypermethylation that causes loss of MLH1 function, loss of function of other genes such as p16, and altered expression of IGF2. Importantly, we demonstrate that the normal colonic mucosa shows aberrant hypermethylation and LOI of IGF2 as a sign of a field defect.

Materials and Methods

Tissue Samples and DNA/RNA Extraction. We randomly selected sporadic CRC patients from whom samples of freshly frozen tumor and matched normal colonic mucosa were available. The tissue from which the normal colonic mucosa was obtained was not immediately adjacent to the tumor. Furthermore, we selected tumor and normal mucosa from HNPCC patients with previously identified germline mutations of MLH1. The MSI status of all tumors had been determined previously (6, 7, 20). Among the sporadic tumors we chose, 51 were MSI(+) and 89 were MSI(−). All 20 HNPCC tumors were MSI(+), and the patients had germline mutation of MLH1 or MSH2 as described (6, 7). All patients gave informed consent before sample collection, according to institutional guidelines. DNA was extracted as described (6). We selected informative samples in which heterozygosity for the IGF2 ApaI polymorphism allowed imprinting analysis by the previously described method (13, 14). RNA was extracted by using TRIZol according to its manufacturer’s instructions (GIBCO/BRL).

Preparation of RNA-Specific PCR Product Containing IGF2 Polymorphism. It is critical to avoid genomic DNA contamination of RNA when examining the imprinting pattern. To eliminate genomic

Abbreviations: LOI, loss of imprinting; RDI, retention of imprinting; IGF2, insulin-like growth factor II; MSI, microsatellite instability; CRC, colorectal cancer; HNPCC, hereditary nonpolyposis colorectal cancer; SNuPE, single nucleotide primer extension; MSP, methylation-specific PCR; DMR, differentially methylated region.

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DNA contamination, total RNA was treated with RNase-free Dnase I (Ambion, Austin, TX) according to its manufacturer’s instructions. After inactivation of Dnase, total RNA was treated with Superscript II (GIBCO/BRL) to produce cDNA, by using random hexamers according to its manufacturer’s instructions. We amplified a RNA-specific PCR product (1.3 kb) containing the IGF2 exon 9 Apa I polymorphism from cDNA by using an exon-connecting primer pair P1 5′-GACACCTCAGTTGCTCTG-3′ (exon 7) and P2 5′-CGGGGATGCTATAAGGTATGAG-3′ (exon 9). This RNA-specific product could be clearly distinguished from the genomic DNA-specific product (3.3 kb) on 1% agarose gels. To further eliminate genomic DNA contamination, the RNA-specific product was excised from the agarose gel after electrophoresis and purified with a QIAEX II gel extraction kit (Qiagen, Chatsworth, CA). Then this RNA-specific product was subjected to a second round of PCR with P3 5′-CTTGGACCTTGTAGCTCAATTTGG-3′ and P4 5′-GGTCTGTCCAAATTTACCTTCA-3′ (20–25 cycles). The second PCR product was treated with exonuclease I (usb, Cleveland) and shrimp alkaline phosphatase (usb) before the primer extension reaction.

Quantitative Analysis of IGF2 Imprinting by Single Nucleotide Primer Extension (SNuPE) Assays. Fluorescent SnuPE assays were carried out in 20 μl containing 50 ng PCR product, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 0.2 pmol 6-carboxyfluorescein-labeled primer, and 0.64 units Thermo Sequenase (Amersham Pharmacia) in the buffer provided by the manufacturer. The extension primer 5′-6-carboxyfluorescein-CTGAAACGCAAGAGAAAAAAGAA-3′ was purchased from Perkin-Elmer. The reaction was carried out in a thermal cycler (Perkin-Elmer) with an initial denaturation step of 2 min at 95°C followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The products of the primer extension reaction were analyzed on an ABI 377 sequencer with a 9% denaturing polyacrylamide gel. The allelic ratios were quantitated with the GENOTYPER software program (Applied Biosystems and Perkin-Elmer) by measurement of the fluorescent intensity of the A allele (26 bp) and G allele (31 bp) products. To investigate the ability of the assay to measure the relative contributions of the two alleles, we tested serial ratio mixtures of the two sequences in the inserts of two plasmids containing each allele of the RNA-specific 1.3-kb PCR products. These plasmid mixtures were subjected to nested PCR and SnuPE assays as described previously.

Methylation Analysis of MLH1 and p16 Promoter Regions. Bisulfite treatment of genomic DNA was carried out as described previously (21). Methylation-specific PCR (MSP) was used for the CpG island region upstream of MLH1 according to the procedure detailed by Herman et al. (2). Moreover, Deng et al. (22) reported that methylation of the CpG sites in another portion of the MLH1 upstream region was highly correlated with MLH1 silencing. This small region was amplified from the bisulfite-treated DNA by the primers 5′-GTAGATATT-TAGTAGGATTATAAGT-3′ and 5′-ACCTTCAAC-CAATACCTCAAT-3′. The 330-bp bisulfite-PCR products were digested by BstUI (New England Biolabs), which cleaves only the CCGG sequence that was unconverted by bisulfite treatment when methylated and yields bands of 203 and 127 bp. The methylation status of p16 was determined by MSP as described (21).

Methylation Pattern of IGF2/H19 Differential Methylated Region (DMR). To investigate the allele-specific methylation of H19 DMR, we selected samples that were heterozygous for three single nucleotide polymorphisms in this region (8008 nt C/A, 8097 nt G/A, 8217 nt C/G; GenBank accession no. AF125183). This region was amplified from normal genomic DNA by using forward primer 5′-GGACGGAATTTGTTGATTT-3′ and reverse primer 5′-AGGCAATTGATCTAGTTAA-3′. This PCR product was directly sequenced by the reverse primer to evaluate its heterozygosity at the single nucleotide polymorphisms. Bisulfite-PCR was carried out on the heterozygous samples [nine LOI tumors, seven imprinted tumors (ROI, retention of imprinting), eight LOI normal mucosa, and six ROI normal mucosa] with the primers 5′-GTAGGTTTTTTGGTAGATATTAGT-3′ and 5′-CAGACAAAACAAACATTACAT-3′. The 500-bp PCR products were cloned into TA vector pCR2.1 (Invitrogen), and at least 20 positive plasmid clones were extracted with a QIAprep Spin Miniprep kit (Qiagen) and sequenced with the ABI sequencing system (Perkin-Elmer and Applied Biosystems).

Statistical Analysis. All comparisons for statistical significance were performed by Fisher’s exact test, with all P values representing two-tailed tests.

Results

Fluorescent SnuPE Assays of Allele-Specific Expression. We adapted a quantitative method, fluorescent SnuPE assay, to analyze allele-specific expression. This assay discriminates between the two alleles and quantitates the relative expression from the two alleles (23). Incorporation of ddATP and dGTP provides a size difference between the primer extension products (26 bp and 31 bp). The heights of the fluorescent peaks on the chromatograms reflect the contributions of the two alleles. We determined the accuracy of quantitation by mixing two plasmids, each containing one of the IGF2 Apa I polymorphisms (A allele or G allele) in known proportions and analyzing the PCR products amplified from the mixtures (Fig. 1). The observed values were close to the expected values, with a correlation coefficient of 0.998, establishing the quantitative nature of the fluorescence-based SnuPE assays.
LOI of IGF2 and MSI Phenotype. Sporadic CRCs and HNPCC tumors with mutations of MLH1 were analyzed for the ApaI polymorphism (A/G) in exon 9 of the IGF2 gene. Among the 160 samples, 62 were heterozygous. RNA from these tumors and matched normal colonic mucosa was subjected to SNuPE assays. The allelic ratios of the fluorescent peaks from the A allele and G allele products were measured (Fig. 2). Many of these specimens showed minimal but detectable expression of the less abundant allele.

The results of the LOI study are summarized in Fig. 3. Of the 20 MSI(1) sporadic CRCs, 17 (85%) exhibited LOI in the tumor tissues. The normal mucosa from these 17 individuals showed LOI in 13 instances; in addition, normal mucosa from two individuals showed LOI, whereas the corresponding tumors did not. In contrast to the sporadic MSI(+) CRCs, none of the five mutation-positive samples with MSI(+) derived from HNPCC patients showed LOI (P < 0.001 versus sporadic MSI-positive), and their normal mucosa showed the normal imprinting pattern. Of 37 sporadic MSI(-) CRCs, 4 (11%) showed LOI in the tumors, and the normal colonic mucosa showed LOI in 6 (16%) (P < 0.001, versus sporadic MSI-positive).

Methylation Status of MLH1 and p16 Promoter Regions. Sporadic MSI(+) CRCs are infrequently mutated in mismatch repair genes (24), but alternatively the CpG sites in the promoter region of MLH1 are hypermethylated, leading to loss of MLH1 expression and resulting in a MSI phenotype (1–5). Two regions upstream of MLH1 are known to be affected by methylation (2, 22). We studied these two regions by the bisulfite-PCR method to investigate the correlation between the methylation and MSI status. As summarized in Fig. 3, by MSP, the CpG island upstream of MLH1 was hypermethylated in 19 of 20 (95%) of the sporadic MSI(+) CRCs. In the more 3' region defined by Deng et al. (22), 16 of 20 (80%) of the sporadic MSI(+) CRCs were methylated. In contrast, 8 of 37 (22%) of the MSI(-) CRCs and 3 of 5 (60%) of the HNPCC tumors showed methylation by MSP. The analysis of the 3' region showed 2 of 37 (5%) in MSI(-) and 0 of 5 (0%) in HNPCC tumors. Thus the methylation of the CpG sites in the 3' region displayed a higher degree of correlation with MSI status than the region examined by MSP and appeared to discriminate more specifically between MSI(+) and MSI(-) CRCs.

The methylator phenotype is known to display a high incidence of p16 promoter methylation (8, 25). To further charac-
We characterized our CRC panel with regard to methylation, we determined the methylation status of p16 by MSP. In the MSI(2) tumors, 11 of 37 (30%) displayed methylation of p16, and, in contrast, in sporadic MSI(1) tumors hypermethylation of p16 was observed in 14 of 20 (70%)(P, 0.05 versus MSI-negative). This result demonstrated a strong link between p16 methylation and MSI.

Methylation Status of the DMR in the IGF2/H19 Locus. The human H19-associated DMR that is located upstream of H19 contains two repeat units, each consisting of a 450-bp repeat (A1 and A2) associated with seven 400-bp repeats (B1–B7), as shown in Fig. 4. These repeats were originally suggested to harbor the paternal methylation imprinting mark of the human H19 gene (26). To investigate whether abnormal methylation in this region was associated with the LOI of IGF2 in CRCs, we selected several tumors showing LOI or ROI that were heterozygous for three single nucleotide polymorphisms (C>T, G>A, C>G) within the DMR, allowing us to discriminate between the two alleles. Methylation was analyzed by the bisulfite-sequencing method. Tumors with ROI as well as the corresponding normal tissue showed monoallele-specific methylation (Fig. 5A). One allele was completely methylated in more than 25 CpG sites in this panel. Moreover, in the MSI(1) CRC panel, comparing the p16 methylation and LOI, all 4 tumors with LOI (4/4) displayed hypermethylation of p16 whereas only 8 of 33 (24%) tumors without LOI displayed p16 hypermethylation (P < 0.001). This result indicated a strong correlation between p16 methylation and LOI, even in the MSI(1) CRCs, and appears to single out a small but consistently hypermethylated subpopulation of tumors.

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region. The other allele was unmethylated or methylated at just one to four sites. In contrast, LOI tumors showed one allele fully methylated and the other allele partially methylated (Fig. 5B). We noted that, in the partially methylated alleles, CpG sites 5–9 were obligatorily methylated in one or several clones. These sites are boxed in Fig. 5. Furthermore, the same sites 5–9 were biallelically methylated in normal colonic mucosa from three of eight patients displaying LOI, whereas none of the tumors or normal colonic mucosa with ROI displayed biallelic methylation at this site (Table 1). As summarized in Table 1, this difference establishes a correlation between the emergence of biallelic methylation in these CpG sites and LOI of IGF2 not only in tumors, but in normal colonic mucosa as well.

The unmethylated DMR has been proposed to act as a chromatin boundary or insulator that blocks the interaction of IGF2 with enhancers that lie 3′ of H19 (27, 28). According to two very recent reports (29, 30), the specific sequence containing the CpG sites 5–9 in the region we examined has been identified as a CTCF-binding site that is likely to be a critical sequence for imprinting control in mice as well as in human beings. This sequence can bind to CTCF protein (31) and block the enhancer activity when unmethylated. Methylation of this sequence was shown to abolish its enhancer-blocking activity, allowing the enhancers access to IGF2 (27, 28). In most of the LOI tumors we examined, the bisulfite-sequencing analysis demonstrated that there were inevitably clones whose CTCF-binding site (CpG sites 5–9) was methylated in both alleles. This methylation can be predicted to abolish the function of this DMR as a chromatin insulator and explains the biallelic expression known as LOI.

Discussion

In this study we examined the relationship between methylation and gene expression in sporadic and inherited CRCs and corresponding normal colonic mucosa. First we designed a fluorescence-based SNuPE assay for analyzing allele-specific expression and found it to be highly accurate and reproducible in assessing allele-specific expression quantitatively. Most authors have used endonuclease restriction digestions for this purpose. However, this is not an ideal method for quantitation, as it is susceptible to errors due to partial digestion, perhaps mainly because of heteroduplexes forming in the PCR products that are refractory to enzyme digestion (32). To overcome these problems, we took advantage of SNuPE assays for the analysis of imprinting status.

Our findings are consistent with those from a previous study (10) and suggest a strong correlation between MSI caused by MLH1 hypermethylation and LOI of IGF2 in sporadic CRCs. We could exclude the hypothesis that deficient mismatch repair or MSI is the determinant of the imprinting status of IGF2, because none of the HNPCC tumors that also displayed MSI showed LOI, whereas a few sporadic tumors without MSI did show LOI.

Herman et al. (2) used MSP to study the promoter region of MLH1 and showed that this methylation was often, but not invariably, associated with loss of expression of MLH1. Moreover, Deng et al. (22) demonstrated that the methylation of CpG sites in a more proximal small region invariably correlated with the absence of MLH1 expression. We examined the methylation status in both regions by bisulfite-PCR. In the majority of MSI(−) sporadic CRCs, the CpG islands in these two regions were hypermethylated, but in some MSI(−) CRCs and HNPCC tumors, the more proximal small region was unmethylated. This observation allows us to conclude that the best correlation between hypermethylation and MSI applies to the small proximal region. A strong link has been reported between MSI and methylator phenotype (8, 33), where the CpG islands in the promoters of certain genes are hypermethylated. It has been postulated that many tumor suppressor genes, including p16 (8, 25, 33), are likely to be silenced by this methylation (34, 35). Because MLH1 is also frequently methylated, an overlap occurs between the MSI and methylator phenotypes. Our results revealed that the majority of the sporadic MSI(+) CRCs with LOI displayed hypermethylation of the promoters of both MLH1 and p16. Moreover, a minority of MSI(−) tumors displayed LOI (4 of 37, 11%), and all of the MSI(−) tumors with LOI displayed p16 methylation, indicating that p16 methylation was strongly associated with LOI, even in MSI(−) tumors. This association suggests specificity of the genes targeted by the methylation in CRCs. We then hypothesized that the methylator phenotype affects not only p16 and MLH1, but also the IGF2/H19 imprinting control region, resulting in LOI, and decided to test the hypothesis.

Table 1. Biallelic methylation of a CTCF-binding site detected by bisulfite sequencing in CRCs and normal mucosa with LOI or with ROI of IGF2  

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Normal mucosa</th>
<th>Total</th>
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<tbody>
<tr>
<td>LOI</td>
<td>6/9 (66%)</td>
<td>3/8 (38%)</td>
</tr>
<tr>
<td>ROI</td>
<td>0/7 (0%)</td>
<td>0/6 (0%)</td>
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Detailed analyses in the mouse have identified a DMR that is located upstream of the H19 gene and is methylated only in the paternal allele (34, 35). H19, which lies downstream of IGF2, displays a reciprocal imprinting pattern with expression from only the maternal allele in almost all tissues (36). Experimental deletion of DMR showed that it plays a crucial role in the regulation of imprinting. “Insulator elements” acting in cis were proposed to explain the effect of this DMR on the reciprocal imprinting pattern of the Igf2/H19 locus (35). The DMR is proposed to serve as an inactivation center when methylated and as a chromatin “insulator” or “boundary element” when unmethylated (34, 35). According to this hypothesis, methylation of the DMR inactivates the insulator elements, making the Igf2 promoter accessible to enhancer elements that are shared by Igf2 and H19. The human IGF2/H19 locus resembles that of the mouse in that a DMR is upstream of the human H19 gene, and allele-specific methylation of the human DMR is associated with the IGF2/H19 imprinting pattern (37), suggesting that LOI of IGF2 in human tumors can be associated with aberrant methylation of the DMR. Actually, Wilms’ tumors with LOI were demonstrated to be biallelically methylated in this region, in contrast to the monoallelic methylation in Wilms’ tumors without LOI (26).

Our results firmly established that CRC samples with ROI showed complete methylation on one allele and low-level or absent methylation on the other, whereas the samples with LOI exhibited complete methylation on one allele and partial methylation on the other, indicating the relaxation of allele-specific methylation. We noticed a core region consisting of five CpG sites (nos. 5–9 in Figs. 4 and 5B) that appeared to be consistently methylated on most tumor alleles that showed partial methylation. We postulated that this core region might be important for the imprinting status. Indeed, while we were writing this paper, crucial results emerged from two studies of the corresponding region in mice (29, 30). Sequences capable of binding to CTCF protein were identified within the DMR, where seven conserved CTCF-binding sites occur in humans. CTCF protein is a highly conserved DNA-binding protein required for the enhancer-blocking activity of insulators (31). The region we examined contained one of the CTCF-binding sites (CpG sites 5–9), and in most of the LOI tumors we examined, they were methylated in both alleles. Our findings in human tumors are highly consistent with the hypothesis that CTCF mediates methylation-sensitive...
 enhancer-blocking activity and controls imprinting of human IGF2/H19. Thus methylation results not only in the MSI phenotype with widespread genetic instability and in the down-regulation of p16 affecting the cell cycle, but in addition can modify cell growth by promoting biallelic expression of IGF2.

The causes of aberrant methylation or methylator phenotype are not known. Imbalance of de novo methylation factors and protecting factors may be important in bringing about aberrant methylation (38, 39). Age-related methylation of some genes was observed in normal colonic mucosa and is thought to constitute a field defect in colon, partially explaining the dramatic correlation between age and advanced CRC incidence (40, 41). A change in methylation might occur by both endogenous genetic factors and exogenous factors, such as carcinogen exposure, and it might affect the genes that regulate the growth or differentiation of colorectal epithelial cells, resulting in a hyperproliferative state or prestige of tumor development. We consider it highly significant that the LOI of IGF2 and aberrant methylation in the CTCF-binding site that we observed in some tumors occurred in the normal colonic mucosa of some patients as well. This finding gives credence to the concept of a generalized field defect in such colons. Whether the LOI might even represent a cancer-prone state, as one might deduce from the results of Cui et al. (10), cannot be determined from studies on patients who have already been affected by cancer. Indeed, prospective studies in which healthy individuals are tested for LOI and/or methylation status, and then followed for extended periods, would have the power to settle this question.

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