DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis

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Cancer cells have aberrant patterns of DNA methylation including hypermethylation of gene promoter CpG islands and global demethylation of the genome. Genes that cause familial cancer, as well as other genes, can be silenced by promoter hypermethylation in sporadic tumors, but the methylation of these genes in tumors from kindreds with inherited cancer syndromes has not been well characterized. Here, we examine CpG island methylation of 10 genes (hMLH1, BRCA1, APC, LKB1, CDH1, p16INK4a, p14ARF, MGMT, GSTP1 and RARβ2) and 5-methylcytosine DNA content, in inherited (n = 342) and non-inherited (n = 215) breast and colorectal cancers. Our results show that singly retained alleles of germline mutated genes are never hypermethylated in inherited tumors. However, this epigenetic change is a frequent second ‘hit’, associated with the wild-type copy of these genes in inherited tumors where both alleles are retained. Global hypomethylation was similar between sporadic and hereditary cases, but distinct differences existed in patterns of methylation at non-familial genes. This study demonstrates that hereditary cancers ‘mimic’ the DNA methylation patterns present in the sporadic tumors.

INTRODUCTION

It is estimated that ~5–10% of cancers occurs with a strong familial association. In colorectal carcinoma, the two most common hereditary predisposition syndromes are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP), caused by germline mutations in DNA mismatch repair genes (mainly hMLH1 and hMSH2) and APC, respectively (reviewed in 1,2). A third, less prevalent, familial colon tumor disease is the Peutz-Jeghers syndrome, caused by germline mutations in the serine/threonine kinase LKB1 (3,4). In sporadic colorectal tumors, the hMLH1, APC and LKB1 genes may undergo transcriptional inactivation by promoter hypermethylation of their respective CpG islands (5–10). hMLH1 hypermethylation is tightly linked in sporadic colon tumors with microsatellite instability (6–8), aberrant methylation of APC is seen in 18% of sporadic colon cancers that lack APC gene mutations (5,9) and LKB1 CpG island methylation is present in a small group of sporadic colon tumors with characteristic pathological features that resemble Peutz-Jeghers syndrome (10). Only a few attempts to clarify the impact of aberrant CpG island methylation of any of these

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Epigenetic changes in cancer are not limited to hypermethylation of gene promoter CpG islands, but also include a simultaneous global demethylation of the genome (5,18–20). Total DNA hypomethylation of the malignant cell has been proposed as a cause for chromosomal instability, reactivation of endogenous viral sequences and up-regulation of certain genes (reviewed in 18,20). No studies examining hereditary cancers for 5-methylcytosine (m5dC) DNA content have been reported.

In the present study, we examined hypermethylation of familial cancer genes, other frequently methylated non-familial genes, and total levels of methylation in both sporadic and inherited forms of breast and colon cancer to determine similarities and differences in epigenetic processes in these alternate pathways of cancer development.

RESULTS

Promoter hypermethylation of tumor suppressor genes as a ‘second hit’ in hereditary tumors

We have previously demonstrated for the four familial genes included in our study, hMLH1, APC, LKB1 and BRCA1, that promoter hypermethylation at these loci analyzed by methylation-specific PCR (MSP) correlates with loss of gene expression (7,9,10,13). Here, we examine how epigenetic inactivation relates to the germline mutational data and LOH status.

Two-thirds of HNPCC tumors show no identifiable second hit (12,21–23) and promoter methylation may be a likely mechanism to accomplish biallelic inactivation. In our study, four of 10 (40%) HNPCC tumors with hMLH1 germline mutations but without LOH at hMLH1 gene were methylated at hMLH1, as opposed to none of the eight hMLH1 germline mutant tumors with LOH (Table 1; Fig. 1). An identical scenario was seen for the methylation status of LKB1 in Peutz-Jeghers colon tumors. In these Peutz-Jeghers families, the LKB1 CpG island was hypermethylated in two of seven (29%) tumors that retained both copies of the gene, whereas no hypermethylation was seen for the LKB1 gene in 13 tumors from Peutz-Jeghers families that had LOH at this locus (Table 1).

In contrast, hypermethylation of the APC gene is extremely uncommon in tumors from FAP families with APC germline mutations. Only one of 84 (1%) samples analyzed was hypermethylated at the APC promoter and that single tumor with aberrant methylation of any other candidate genes in the hereditary breast tumors have been reported.

Table 1. Promoter hypermethylation as a second ‘hit’ in hereditary human cancer

<table>
<thead>
<tr>
<th>CpG island</th>
<th>Genes</th>
<th>BRCA1</th>
<th>hMLH1</th>
<th>APC</th>
<th>LKB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Status</td>
<td></td>
<td>LOH</td>
<td>RET</td>
<td>LOH</td>
<td>RET</td>
</tr>
<tr>
<td>Methylated</td>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unmethylated</td>
<td></td>
<td>21</td>
<td>1</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

LOH, presence of loss of heterozygosity in the tumor; RET, retention of both alleles in the tumor.
**Table 2. CpG island aberrant methylation in sporadic and hereditary human cancer**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Frequency of hypermethylation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon tumors</td>
<td></td>
</tr>
<tr>
<td>Sporadic (n = 109)</td>
<td>p16(^{INK4a}) 55 (38/109)  p14(^{ARF}) 33 (36/109)  MGMT 36 (39/109)  APC 17 (19/109)  hMLH1 9 (10/109)  LKB1 6 (6/109)</td>
</tr>
<tr>
<td>FAP (n = 84)</td>
<td>20 (17/84)  20 (17/84)  11 (9/84)  1 (1/84)  4 (3/84)  0 (0/84)</td>
</tr>
<tr>
<td>HNPCC (n = 70)</td>
<td>19 (13/70)  19 (13/70)  24 (17/70)  13 (9/70)  24 (17/70)  3 (2/70)</td>
</tr>
<tr>
<td>Peutz-Jeghers (n = 26)</td>
<td>15 (4/26)  0 (0/26)  0 (0/26)  0 (0/26)  4 (1/26)  4 (1/26)</td>
</tr>
<tr>
<td>Breast tumors</td>
<td></td>
</tr>
<tr>
<td>Sporadic (n = 106)</td>
<td>p16(^{INK4a}) 15 (16/106)  CDH1 19 (20/106)  RAR(\beta) 24 (25/106)  GSTP1 24 (25/106)  BRCA1 12 (13/106)</td>
</tr>
<tr>
<td>BRCA1 families (n = 99)</td>
<td>18 (18/99)  21 (21/99)  17 (17/99)  4 (4/99)  6 (6/99)</td>
</tr>
<tr>
<td>BRCA2 families (n = 34)</td>
<td>26 (9/34)  6 (2/34)  6 (2/34)  9 (3/34)  9 (3/34)</td>
</tr>
<tr>
<td>BRCAX families (n = 29)</td>
<td>14 (4/29)  7 (2/29)  24 (7/29)  14 (4/290)  3 (1/290)</td>
</tr>
</tbody>
</table>

**BRCA1 mutant families that retained both alleles, one was methylated at the BRCA1 CpG island (Table 1; Fig. 1). Thus, BRCA1 promoter hypermethylation may play a role as a second event of inactivation in BRCA1 families, but this mode of inactivation is infrequent due to the dominance of genetic deletions as ‘second hits’.

In total, for these genetic forms of breast and colon cancer, 0 of 44 alleles in tumors with LOH at the respective loci showed hypermethylation of the promoter region, whereas this change was present in eight of 26 tumors (31%, \(P < 0.0001\)) in which both copies of the genes were retained (Table 1). This emphasizes the non-stochastic selective advantage of this epigenetic change in that a second hit is not required on a singly retained mutated allele to achieve functional ablation of the gene. Intriguingly, for all of the germline mutation genes examined, there still remains a subset of tumors without an apparent second ‘hit’ involving either LOH or methylation. In these cases, a dominant effect of the germline mutation or a somatic mutation may be present, as demonstrated for CDH1 (25) and APC (2,24).

**Profile of CpG island hypermethylation in hereditary breast and colon tumors**

Genes other than the classic tumor suppressor genes hMLH1, APC, LKB1 and BRCA1 can also undergo inactivation by promoter hypermethylation. To look at this epigenetic change in sporadic and inherited tumors, we examined genes frequently methylated in colon and breast cancers. For each gene, we have previously shown that hypermethylation at these sites correlates with gene silencing and that the silencing can be partially relieved by demethylation of the promoter region (18,20).

Tumors occurring in the setting of FAP and HNPCC share many of the same epigenetic changes as sporadic colorectal cancer, albeit at slightly decreased frequency (Table 2). For the cell-cycle inhibitor p16\(^{INK4a}\), the MDM2-p53 regulator p14\(^{ARF}\) and the DNA repair O6-methylguanine DNA methyltransferase (MGMT) genes, the percentage of FAP and HNPCC tumors with promoter region methylation was different (\(\chi^2, P = 0.01\)) from sporadic colorectal tumors. The only exception was MGMT methylation in HNPCC tumors which occurred at the same frequency as in sporadic tumors (\(\chi^2, P = 0.11\)) (Table 2; Fig. 2). The combination of mismatch repair deficiency and MGMT inactivation has been observed in cancer cells leading to tolerance to alkylating agents and high mutational rates (26).

Similar enhanced mutagenicity can be invoked in one case that had germline hMSH2 mutation and hMLH1 hypermethylation. Simultaneous disruption of two mismatch repair genes has been described by Malkhosyan et al. (27). In contrast to FAP and HNPCC tumors, Peutz-Jeghers tumors demonstrated relatively few epigenetic changes at these genes, except p16\(^{INK4a}\) methylation (Table 2; Fig. 2). This lack of epigenetic similarity is consistent with the different pathological phenotype of this tumor.

Unlike colorectal cancer where the patterns of epigenetic alterations were similar but at lower frequency in familial tumors, in breast cancer we find that tumors occurring in the familial setting have distinct differences. BRCA1 tumors resembled sporadic breast cancer for methylation frequencies of p16\(^{INK4a}\), CDH1 and retinoic acid receptor \(\beta\)-2 (RAR\(\beta\)) (Table 2; Fig. 3), but had a much lower frequency of glutathione S-transferase P1 (GSTP1) methylation (24 versus 4%; \(\chi^2, P = 0.0001\)). In contrast, BRCA2-associated tumors had a slightly higher frequency of p16\(^{INK4a}\) methylation (26 versus 15%; \(\chi^2, P = 0.13\)) and lower frequencies of methylation of the other genes (\(\chi^2, P = 0.02-0.07\)) (Table 2; Fig. 3). We had three BRCA2 breast tumors that also displayed BRCA1 aberrant methylation (Fig. 3). Simultaneous LOH of both BRCA1 and BRCA2 loci happens in sporadic breast tumors and a de novo BRCA1 mutation has been found in a BRCA2 patient (28). In our last group, BRCAX tumors, the epigenetic profile appears to be a merger of BRCA1 and BRCA2 patterns (Table 2; Fig. 3), though the sample size is limiting.

**Global genomic hypomethylation in hereditary tumors**

We also analyzed whether the decrease in overall genomic DNA methylation seen in sporadic forms of cancer (29,30) occurs in familial forms of tumors. This generalized loss of m\(^{3}\)C in malignant cells occurs mainly in the CpGs scattered in the ‘bodies’ of genes and also in repetitive sequences (29,30).

In our series, the m\(^{3}\)C DNA content, analyzed by high performance capillary electrophoresis (HPCE), was 30 and 56% lower for sporadic colorectal (n = 26) and breast (n = 13) tumors, respectively, as compared to normal tissues (34 normal colon plus 15 normal breasts) (Fig. 4). Colorectal and breast cancer cell lines (n = 9) also had similar values of m\(^{3}\)C loss.
and Drosophila melanogaster D.Mel 2 cells only showed minimal vestiges of m\(^3\)dC, as expected (31) (Fig. 4). The hereditary tumors studied from FAP \((n = 19)\), HNPCC \((n = 13)\), BRCA1 \((n = 15)\) and BRCA2 \((n = 9)\) patients also displayed global hypomethylation with average decreases of their m\(^3\)dC DNA content of 43, 42, 42 and 30%, respectively (Fig. 4). These values, do not differ statistically between hereditary tumors and their sporadic counterparts \((\chi^2, P > 0.05)\). Thus, hereditary human tumors undergo a global hypomethylation of their genomes as sporadic tumors do.

**DISCUSSION**

Our results confirm in a systematic fashion using one of the largest collections of hereditary tumor samples that CpG island promoter hypermethylation may be considered a ‘bona fide’ mechanism to accomplish ‘second hits’ for the inactivation of tumor suppressor genes in families. Clues for this scenario had previously been reported in studies of CDH1, VHL and hMLH1 aberrant methylation in hereditary diffuse gastric cancer, familial renal cancer and HNPCC tumors, respectively (11,12,25,32). Our findings expand these early observations and highlight the selective advantage of epigenetic gene silencing. Our results show that aberrant methylation of the CpG island is never present when only a mutant allele is present in the tumor. If two alleles are present and the tumor has a germline mutation, promoter methylation may accomplish the biallelic inactivation of that particular gene acting on the retained wild-type allele. This data also agrees with our previous description of the \(p16^{INK4a}\) methylation status in a colorectal cancer cell line that retains two alleles of \(p16^{INK4a}\): one allele is mutant and unmethylated and the other is wild-type and hypermethylated (33). In the familial tumors, the importance of epigenetic inactivation depends on the gene. Tumors with germline mutations in \(APC\) or \(BRCA1\), because they have frequent ‘genetic’ second-hits, somatic mutations and LOH, respectively, have little ‘room’ for methylation. In HNPCC and Peutz-Jeghers tumors, more ‘epigenetic’ second-hits may be observed.

We also found that hereditary tumors demonstrate other epigenetic alterations, CpG island hypermethylation of other tumor suppressor genes and global hypomethylation, in a similar extent to non-familial tumors. These findings agree with the data from the studies of molecular alterations in familial colorectal and breast tumors that show that they also harbor similar alterations in oncogenes (\(K\)-ras, \(MYB\)) or tumor suppressor genes (\(p53\), TGF\(\beta\)RII, LOH at different loci, etc.) as the sporadic forms do. Thus, familial tumors are ‘pushed’ through
the tumorigenic pathway due to their initial germline mutation, but other genetic and epigenetic lesions are also necessary.

In conclusion, our results suggest that epigenetic changes in familial breast and colon cancer are similar to the sporadic forms of these cancers, but with some qualitative and quantitative differences. Overall methylation levels are comparable, but methylation of certain CpG islands differs between sporadic and inherited forms of cancer. Subsequent to genetic initial alterations of 'gatekeeper' and 'caretaker' genes, such as BRCA1, hMLH1 and APC, the progression of both sporadic and inherited tumors appears to share and need an altered state of DNA methylation. Most notably, hypermethylation of gene promoters can frequently play a direct role in the silencing of wild-type genes either as a primary or second hit in both genetic and sporadic forms of the disease.

MATERIALS AND METHODS

Tumor samples and mutational characterization

The set of sporadic colorectal and breast tumors samples analyzed is described in part by Esteller et al. (5,9,13). Additional cases of sporadic tumors were obtained from the Hospitals Duran i Reynals and Clinica Dexeus, Barcelona, Catalonia, Spain and The Johns Hopkins Oncology Center. The study protocols were approved by the corresponding Ethics Committees. All the samples were frozen in liquid nitrogen immediately after resection and stored at –80°C until processing. DNA was extracted by standard methods. Analysis of germline mutations and loss of heterozygosity in hMLH1, hMSH2, APC, LKB1, BRCA1 and BRCA2 in the familial tumors is described elsewhere (3,9,11,16,34–40). In BRCA1 (n = 99) and BRCA2 (n = 34) tumors, each sample was obtained from an independent patient. In all these 123 cases, a germline mutation in BRCA1 or BRCA2 was found. Among the familial colorectal tumors, 70 HNPCC tumors were obtained from 66 independent patients, 84 FAP tumors from 25 independent patients, and 26 Peutz-Jeghers tumors from six independent patients. According to the germline mutational characterization, 61 of 66 HNPCC patients had a demonstrable mutation in hMLH1 or hMSH2, 19 of 25 FAP patients had a mutation in APC and all six Peutz-Jeghers patients had a mutation in LKB1.

Analysis of CpG islands methylation status

DNA methylation patterns in the CpG islands of each gene were determined by chemical modification of the unmethylated,
but not the methylated, cytosines to uracil, and subsequent PCR using primers specific for either the methylated or the modified unmethylated DNA. The primers and PCR conditions for the MSP analysis have been previously described for p16\(	ext{INK4a}\), p14\(\text{ARF}\), APC, MGMT, LKB1, hMLH1, BRCA1, GSTP1 and CDH1 (5,7,9,10,13). In all cases the presence of methylation at these CpG sites has been correlated with loss of gene expression (5,7,9,10,13). Primer sequences for \(\text{RAR}\beta2\) were for the unmethylated reaction 5′-ACT CAA CCA ATC CAA CCA AAA CAA-3′ (upper primer) and 5′-GAA TGT GAG TGA TTT-3′ (lower primer). The annealing temperature was 60°C. Placental DNA treated \textit{in vitro} with SssI methyltransferase was used for positive control for methylated alleles and DNA from normal lymphocytes was used as negative control for methylated alleles. A 12 µl aliquot of each PCR reaction was directly loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

**Determination of methylcytosine (mC) content**

The m\(\text{dC}\) content in normal and tumoral DNA from colorectal and breast tumors was determined by HPCE as described by Fraga \textit{et al.} (41). One microgram of DNA was incubated in 20 µl 88% (v/v) formic acid at 140°C for 90 min. After hydrolysis, the samples were reduced to dryness by speed-vac concentration (Savant SC-200). Finally, the dried hydrolyzed samples were re-dissolved in 2 µl H\(\text{O}\) Milli-Q grade and stored at −20°C until their analysis. An uncoated fused-silica capillary (Waters Chromatography S.A.) (600 × 0.075 mm i.d., effective length 540 mm) was used in a capillary electrophoresis system (Capillary Ion Analyzer; Waters Chromatography S.A.) connected to a processing data station Millennium\(\text{20}\) (Waters Chromatography S.A.). The running buffer used was 24 mM NaH\(\text{CO}_3\), pH 9.6 plus 36 mM SDS. The running condition was at 25°C and an operating voltage of 20 kV. On-column absorbance was monitored at 256 nm. Before each run, the capillary was conditioned by washing with 1 mM NaOH for 1 min, followed by 0.1 M NaOH for 3 min and equilibrated with the running buffer for 3 min. Buffers and washing solutions were prepared with Milli-Q water and filtered through 0.45 µm pore size filters. Hydrolyzed samples were injected hydrostatically at 9.8 cm for 15 s, having been previously filtered through 0.45 µm pore filters. Three replicate analyses of each sample were performed and the quantification of the relative methylation in the DNA samples was calculated as the percentage of the mC of the total cytosines (C), calculated as follows: mC peak area × 100/(C peak area + mC peak area).

**REFERENCES**


