DNA Hypomethylation and Ovarian Cancer Biology

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ABSTRACT

Hypomethylation of some portions of the genome and hypermethylation of others are very frequent in human cancer. The hypomethylation often involves satellite 2 (Sat2) DNA in the juxtacentromeric (centromere-adjacent) region of chromosome 1. In this study, we analyzed methylation in centromeric and juxtacentromeric satellite DNA in 115 ovarian cancers, 26 non-neoplastic ovarian specimens, and various normal somatic tissue standards. We found that hypomethylation of both types of satellite DNA in ovarian samples increased significantly from non-neoplastic toward cancer tissue. Furthermore, strong hypomethylation was significantly more prevalent in tumors of advanced stage or high grade. Importantly, extensive hypomethylation of Sat2 DNA in chromosome 1 was a highly significant marker of poor prognosis (relative risk for relapse, 4.1, and death, 9.4) and more informative than tumor grade or stage. Also, comparing methylation of satellite DNA and 15 other gene regions, which are often hypermethylated in cancer or implicated in ovarian carcinogenesis, we generally found no positive or negative association between methylation changes in satellite DNA and in the gene regions. However, hypermethylation at two loci, CDH13 (at 16q24) and RNRI (at 13p12), was correlated strongly with lower levels of Sat2 hypomethylation. The CDH13/Sat2 epigenetic correlation was seen also in breast cancers. We conclude that satellite DNA hypomethylation is an important issue in ovarian carcinogenesis as demonstrated by: (a) an increase from non-neoplastic tissue toward ovarian cancer; (b) an increase within the ovarian cancer group toward advanced grade and stage; and (c) the finding that strong hypomethylation was an independent marker of poor prognosis.

INTRODUCTION

In the United States and Europe, epithelial ovarian cancer causes more deaths than does cancer in any other female reproductive organ. It is estimated that there are ~25,400 new cases of ovarian cancer and 14,300 deaths in the United States (1). Because of the lack of early detection strategies, many ovarian cancer patients present with advanced stage disease, and the overall 5-year survival for these women is <30% (2, 3). Despite the development of new therapeutic approaches, these survival statistics have remained largely unchanged for many years. The most important prognostic parameters for this disease are age, stage, grade, and optimal cytoreductive surgery (where all of the visible cancer in the peritoneal cavity is removed). Clearly, there is a need for a better understanding of the molecular pathogenesis of ovarian cancer so that new drug targets or biomarkers that facilitate early detection can be identified.

Molecular genetic analyses of ovarian cancers have uncovered genetic alterations of several genes, such as c-ERB-B2, c-MYC, and P53, in an appreciable fraction of tumors (4). Global studies of genom rearrangements suggest that changes in additional genes are involved in ovarian tumor progression and are correlated with clinical parameters to a variable extent (5). Microarray expression analysis has revealed candidate marker genes for ovarian epithelial carcinomas (6, 7).

Studies have begun addressing also the epigenetic components of ovarian carcinogenesis (8–12). Changes in DNA methylation status (predominantly at CpG) are among the most common molecular alterations in human neoplasia (13, 14). Often, the aberrant methylation of CpG islands overlapping the promoter region of various genes in cancers has been correlated with a loss of gene expression, and it appears that DNA methylation provides an alternative pathway to gene deletion or mutation for the loss of tumor suppressor gene function (14, 15). DNA methylation changes promise also to be important screening markers for carcinogenesis (16).

Not only is the well-studied hypermethylation of promoter and 5’ gene sequences associated with carcinogenesis very frequently, but also, hypomethylation of certain other parts of the genome is a common cancer-associated phenomenon (15, 17, 18). Furthermore, the extent of hypomethylation often exceeds that of hypermethylation resulting in a net loss of 5-methylcytosine in the DNA (15, 18). This cancer-linked genomic hypomethylation frequently involves long regions rich in satellite 2 DNA sequences (Sat2) in the juxtacentromeric (centromere-adjacent) heterochromatin of chromosomes 1 and 16, which are methylated highly in various normal postnatal somatic tissues (19, 20). It was shown previously that there is significantly more of this hypomethylation in Sat2 in the juxtacentromeric heterochromatin of chromosome 1 (Chr1 Sat2) or chromosome 16 in ovarian carcinomas compared with borderline malignant ovarian tumors [low malignant potential (LMP) tumors] and cystadenomas (21) and that global DNA hypomethylation increases also with the degree of malignancy in these ovarian epithelial neoplasms (22).

In the present study, 115 ovarian cancers and 26 non-neoplastic ovarian specimens were analyzed for hypomethylation at Chr1 Sat2 and for hypomethylation in the major DNA component of all of the human centromeres, satellite α (Satα). Most of these specimens were examined also for hypermethylation in the 5’ regions of 15 different genes that may be involved in ovarian carcinogenesis or that often show cancer-linked hypermethylation. In this analysis, we addressed the following four major issues: (a) the extent of satellite DNA hypomethylation in cancer versus normal tissues; (b) the association of this DNA hypomethylation with important clinicopathological features; (c) the extent of this hypomethylation and the impact on survival of patients who underwent optimal cytoreductive therapy; and (d) the association of satellite DNA hypomethylation with hypermethylation in the above-mentioned 15 gene regions.

MATERIALS AND METHODS

Patients and Samples. Tumor specimens were from a tissue bank and had been collected prospectively from patients operated for gynecological cancers.
at the Department of Obstetrics and Gynecology, Innsbruck University Hospital (Innsbruck, Austria). Clinical, pathological, and follow-up data were stored in a database in accordance with hospital privacy rules. Tumor samples and clinical data were collected with the consent of patients. Part of the specimens were quick-frozen immediately after resection and stored at −80°C until Ilyophilization. The 115 ovarian cancer patients for this study were treated at Innsbruck University Hospital between 1989 and 2000 and staged according to the International Federation of Gynecology and Obstetrics system (Table 1). A platinum-based chemotherapy after surgery was part of the treatment for all but 21 cancer patients (7, 11, 2, and 1 patients, who had LMP, International Federation of Gynecology and Obstetrics I, II, and III ovarian cancer, respectively). After primary treatment, all of the patients were followed at our department at intervals increasing from 3 months to 1 year until death or the end of the study. Follow-up information was available for all of the patients. Routine examinations including systemic review, tumor-marker testing (CA 125), pelvic examination, chest X-ray, pelvic computer tomography, or magnetic resonance imaging were performed to evaluate disease outcome, which was classified as progression-free, relapse, or death according to the WHO criteria for clinical response. A control group consisted of 26 normal ovarian tissues (whole ovary or benign cyst of the ovary) from noncancer patients (Table 1). In addition, DNA from 43 breast cancer specimens (32 invasive ductal, 6 invasive lobular, and 5 otherwise differentiated) were used to look for the type of associations between hypo- and hypermethylation found in ovarian cancer specimens.

DNA Isolation and Methylation Analysis. Genomic DNA from lyophilized, quick-frozen ovarian cancer specimens were isolated using the QIAamp tissue kit (Qiagen, Hilden, Germany). Southern blot analysis with a CpG methylation-sensitive restriction endonuclease, BstBI, was done to assess the extent of hypomethylation in satellite DNA (0.5 μg) under high-stringency (Ch1 Sat2 and Ch1 Sat1) or low-stringency (Sat1 in the centromeres throughout the genome) conditions with a 1.77-kb Ch1 Sat1 (23) or a 1.9-kb Ch1 Sat1 (24) probe as described previously (19). In this study, however, we used a scale of 0, 1, 2, 3, or 4 for hypomethylation scores. For scoring Ch1 Sat2 and Ch1 Sat1 hypomethylation, we used two criteria. The first was the phoshorimagher analysis to determine the ratio of the total <4-kb hybridization signal to that of the total >4-kb signal in each sample divided by the average from the analogous ratios for 4–5 normal postnatal somatic tissues in the same blot (R value). The second was looking for increases in intensity of specific low-molecular-weight bands relative to high-molecular-weight signal in the same lane and comparing tumor samples with sperm DNA, the hypomethylated standard (19, 20) in each blot (Figs. 1–3). Only samples displaying increases in specific, expected sized low-molecular-weight bands relative to the high-molecular-weight signal in the same lane were scored as hypomethylated. Ch1 Sat2 hypomethylation scores for tumors, in italics, followed by typical R values were as follows: 0, <1.8; 2, 2.6–5.0; 3, 5.1–8.0; and 4, >8.0 compared with sperm with a R value of ∼25. For Ch1 Sat1 hypomethylation, hypomethylation scores followed by typical R values for tumors were as follows: 0, <1.4; 1, 1.4–1.8; 2, 1.9–2.7; 3, 2.8–3.9; and 4, >4.0 compared with sperm with a R value of ∼7. For Sat1 in the centromeres throughout the genome, hypomethylation was scored just by assessing band patterns in the X-rays as described in the legend to Fig. 1. We did not use R values for this satellite, because there was much hybridizing DNA in the very high-molecular-weight region, even for sperm DNA. This was probably because of the heterogeneity in Sat1 sequence, including at the BstBI sites, among different centromeres. All of the DNAs used in the analysis were checked for their integrity by examining the ethidium bromide-induced fluorescence in the gel before transfer to ascertain that most of the fluorescence (most of the total DNA) was high molecular weight. Only a few DNA samples displayed substantial degradation, and these were eliminated from this study. Also, three samples displaying no Ch1 Sat2 hypomethylation and three with a large extent of such hypomethylation (score 3 or 4) were analyzed for low-molecular-weight versus high-molecular-weight signal in digestes with CpG methylation-insensitive enzymes and a moderately repeated DNA sequence (DA24) as a blot hybridization probe. Comparison of the ratios of the phosphorimagher signal in low-molecular-weight bands versus in the high-molecular-weight region for each of these samples confirmed that there was no association of DNA degradation with samples displaying satellite DNA hypomethylation.

Sodium bisulfite conversion of genomic DNA and the MethyLight assay were performed as described previously, and PMR (percentage of fully methylated reference) values were determined (25–27). For methylation analysis, two PMR values were calculated separately for the reference genes ACTB and COL2A1, and the average was used. Most of the primers and probes for the MethyLight reactions have been published (28). The forward and reverse primer and the probe, respectively, for the genes unpublished thus far are as follows: RNR1, CGGAGTTGGATACCGGTCG, AAAACAGCGGACCGAAGA, 6FAM-ACGCCCGGTACCACACGCAA-BHQ-1; MCI, TTTCG- GGTGCTTTGTGTATTAGG, ACTACAAATATCAAGTACAGCAACT, 6FAM-TGGCAATAAAGCAGTAAACCCCAACGAA-BHQ-1; TNFRS-F2, GCCGAATCTACGCGGATAGA, ACTTACATTACCTCAGCAGGA, 6FAM-GGCCAAAAACTCTCCTCAGTCTCTTG-BHQ-1, and IGSF4, GG GTTCGGAAGGATGTAGTAAACGCTC, CACTAAAAATCGCTGACAACAC, 6FAM-ACACTGCGCATACTGCAAACCCTCTC-AA-BHQ-1.

Statistical Analysis. Differences of hypomethylation scores between non-neoplastic and cancer specimens were assessed using the Mann-Whitney U test. For additional analysis, we used the highest level of DNA hypomethylation detected in non-neoplastic ovaries as a cutoff level (score 2) and dichotomized cases with methylation scores of ≤2 and >2. All analyses were used two criteria. The first was increased satellite DNA hypomethylation as assessed by high-performance liquid chromatography analysis of DNA digested to deoxynucleosides (19, 21). Many of the tumor samples displayed hypomethylation of the examined satellite DNAs as illustrated in Fig. 1. Satellite DNA hypomethylation in the ovarian samples was scored on a scale of 0–4 relative to various normal postnatal somatic tissue standards, assigned a score of 0, and sperm, assigned the maximal score of 4.

There was a highly significant difference in the levels of satellite methylation between the ovarian cancers and the non-neoplastic ovarian tissues for Ch1 Sat 2, Ch1 Sat1, and Sato throughout the centromeres (Mann-Whitney U test; P < 0.001 for all three regions; Table 1). Relative to normal postnatal somatic tissue standards (Fig. 1), none of the non-neoplastic ovarian specimens had a hypomethylation score of >1 (on a 0–4 scale) for Ch1 Sat2, and only 4% (1 sample) had a hypomethylation score >1 for Ch1 Sator Sato throughout the centromeres (Table 1). However, 12, 43, and 84% of the non-neoplastic ovarian specimens (most of which were normal,
DNA hypomethylation levels have been numbered 0 to 4. Ovarian cancer cases have been ranked with the increase in stage and grade.

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whole ovaries) displayed a slight amount of Chr1 Sat 2, Chr1 Sat/H9251, or general Sat/H9251 hypomethylation relative to tissues from seven diverse somatic organs, which had very similar blot hybridization patterns (Fig. 1; data not shown). This small amount of satellite DNA hypomethylation might reflect the special cellular composition of this organ. We grouped ovarian cancer specimens into two hypomethylation score categories: 2 (score 0, 1, or 2, indicating no, slight, or only moderate hypomethylation, respectively); and 3 (score 3 or 4, indicating much or extreme hypomethylation, respectively). Of the 115 ovarian cancer specimens, 30%, 33%, and 15% of the samples demonstrated hypomethylation scores \( \geq 2 \) for Chr1 Sat 2, Chr1 Sat, or Sat throughout the centromeres, respectively. Hypomethylation of all of the three categories of satellite DNA strongly correlated with each other \( P < 0.0001 \).

### DNA Hypomethylation Markers in Neoplastic Ovarian Tissue Specimens in Relation to Clinicopathologic Features

Using the above-described scoring system for satellite DNA hypomethylation, we looked for associations between this hypomethylation and age, tumor stage, tumor grade, histology, and whether there was tumor remaining after surgery. No significant association was found between

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<td>57</td>
<td>yes</td>
<td>IV</td>
<td>END</td>
<td>III</td>
<td>yes</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Age, age of the corresponding patient in years; Cancer, yes if ovarian cancer and no if non-neoplastic ovary; FIGO, Fédération Internationale des Gynécologues et Obstétristes tumor stage I–IV; Histo, histology; CYS, benign cyst of the ovary; OVA, normal ovary; SER, serous cancer; MUC, mucinous cancer; END, endometrioid cancer; LMP, low malignant potential tumor; MG, tumor grade I–III; RemTu, remaining tumor after surgery; ND, not determined because of technical problems with the blot and the availability of only a small amount of DNA; NA, not assessed; Sat2, Sat, All c, DNA hypomethylation levels at Chr1 Sat2, Chr1 Sat, and Sat throughout the centromeres, respectively, on a scale of no hypomethylation (0) to maximum observed hypomethylation (4), as illustrated in Fig. 1.*
DNA HYPOMETHYLATION AS A PROGNOSTIC MARKER

To assess whether satellite DNA hypomethylation in the cancers was associated with the outcome for the patients, we calculated relapse-free survival and overall survival in ovarian carcinoma patients who received optimal cytoreductive surgery (meaning that no visible tumor had been left in the abdomen after surgery) and excluded the LMP tumor patients, who are known to have a better prognosis. In this subset of 45 patients, univariate analysis revealed no prognostic significance (nor for relapse-free nor for overall survival) for tumor stage, grade, or histology. Only age demonstrated an impact on overall survival (log-rank \( P = 0.008 \); Kaplan-Meier curves not shown).


dna hypomethylation of chr1 sat2 is a prognostic marker independent from classical prognostic markers, we used the Cox multiple-regression analysis that included tumor stage, grade, age, and hypomethylation status of chr1 sat2. a high level of hypomethylation at this region, independently from other parameters, was associated strongly with poor relapse-free survival as well as with poor overall survival. The relative risk for relapse was 4.1 (table 3a) and death was 9.4 (table 3b).

dna hypomethylation and hypermethylation in ovarian cancer. in 96 tumors, we analyzed the CpG-rich promoter or 5’ transcribed regions of 15 genes that either have been shown to play a role in ovarian carcinogenesis or are known to be hypermethylated in various cancers. We were looking for an association between gene region hypermethylation and satellite DNA hypomethylation. Associations (ranked by their strength) between gene hypermethylation and hypomethylation at chr1 sat2, chr1 sat2, or sat2 throughout the centromeres are shown in Table 4a. Hypermethylation of CDH13, a cadherin family gene at 16p24, showed a significant negative association with hypomethylation of all three categories of satellite DNA (Fig. 3). Similarly, methylation of the multicopy RNR1 rRNA locus at 13p12 was associated significantly and negatively with Chr1 Sat2 hypomethylation. In contrast, CALCA hypermethylation was associated positively with Chr1 Sat2 hypomethylation.

Because the finding of two gene regions displaying more frequent hypermethylation in tumors that had no Chr1 Sat2 hypomethylation or lower levels only of this hypomethylation was surprising, we used a different tumor entity, namely breast cancer, to test independently this correlation. The same type of analysis was done on 43 breast cancer specimens. Again, CDH13 hypermethylation was associated with a lesser extent of DNA hypomethylation of Chr1 Sat2 and Sat2 throughout the centromeres, whereas the same trend was seen for RNR1 and the examined satellite DNAs (Table 4b; Fig. 3). No consistent association was found for CALCA hypermethylation and satellite DNA hypomethylation in the breast cancers.

**DISCUSSION**

For diverse cancers, it has been shown that the overall 5-methylcytosine content of the genome and methylation at satellite DNA sequences decreases frequently, although focal de novo methylation at many CpG island overlapping promoters of tumor suppressor genes
increases (15, 16, 18, 21, 22). There are only a few published studies (30, 31) investigating the extent of hypo- and hypermethylation in the same tumor specimens using quantitative methods. Here, we used Southern blot analysis with a CpG methylation-sensitive restriction endonuclease, $B_{sr}BI$, to assess the level of hypomethylation in satellite DNA of ovarian cancer specimens from 115 patients for whom we had an extensive collection of clinical data. We looked for biological correlates of cancer-associated DNA hypomethylation. From three lines of evidence, our study indicates that DNA hypomethylation is an important condition in ovarian cancer. Firstly, hypomethylation in all of the examined types of satellite DNA sequences (juxtacentromeric Sat2 in Chr1, the adjacent centromeric SatO in Chr1, and SatO DNA throughout the centromeres) increased from non-neoplastic ovarian tissue and a variety of normal postnatal somatic tissues toward ovarian cancer. The Sat2 results confirm those from a previous study of 8 ovarian carcinomas, 5 LMP tumors, and 4 cystadenomas (21). Secondly, an increase in DNA hypomethylation within the ovarian cancer group with advanced grade and stage was observed. Thirdly, high levels of DNA hypomethylation were an independent marker of a poor prognosis in a subset of patients who received optimal surgical cytoreductive therapy.

It had been reported previously by Itano et al. (32) that hypomethylation of either of two moderate copy number tandem repeats (one present in several chromosomes in pericentromeric or acrocentric short-arm regions and the other at 8q21) is associated significantly with the postoperative occurrence of hepatocellular carcinoma. However, in that study, DNA hypomethylation was not shown to be linked also to tumor grade or stage. Our investigation of ovarian carcinomas demonstrates such an association between the much more abundant satellite DNA repeats and tumor grade, tumor stage, and relapse-free survival. Therefore, hypomethylation of tandem DNA repeats as well hypermethylation of gene regions, e.g., at APC in primary non-small cell lung cancer, is associated with poor survival in ovarian cancer.
DNA HYPMETHYLATION IN OVARIAN CANCER

Table 2. Association of satellite DNA hypomethylation with clinicopathological features of ovarian cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chr1 Sat2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Chr1 Sato&lt;sup&gt;b&lt;/sup&gt;</th>
<th>All centromeres&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤2 (n = 80)</td>
<td>&gt;2 (n = 35)</td>
<td>≤2 (n = 74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≤2 (n = 95)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60 yr</td>
<td>40</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>&gt;60 yr</td>
<td>40</td>
<td>23</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
</tr>
<tr>
<td>Stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Figo I/II</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Figo III/IV</td>
<td>48</td>
<td>29</td>
</tr>
<tr>
<td>Grading</td>
<td>MG I</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>MG II</td>
<td>48</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>MG III</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Histology</td>
<td>LMP</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Serous</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>33</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Endometrioid</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Remaining tumor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No</td>
<td>41</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>34</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data for carcinomas are shown; 0, 4 and 3 measures, respectively, are missing. Samples with satellite hypomethylation scores of ≤2 had no, slight, or only moderate hypomethylation. Samples with satellite hypomethylation scores of >2 had much hypomethylation.

<sup>b</sup> Information about stage and remaining tumor is missing in 1 and 5 cases, respectively.

<sup>c</sup> FIGO, Fédération Internationale des Gynaecologistes et Obstetristes; LMP, low malignant potential.

Table 3. Multivariate analysis for relapse-free and overall survival of the 45 non-LMP<sup>a</sup> ovarian cancer cases with optimal surgical cytoreductive therapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk of relapse (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60 yr vs. ≤60 yr</td>
<td>1.4 (0.4–4.8)</td>
<td>0.55</td>
</tr>
<tr>
<td>Figo stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III/IV vs. I/II</td>
<td>1.2 (0.3–4.7)</td>
<td>0.81</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III vs. I/II</td>
<td>2.0 (0.5–7.7)</td>
<td>0.31</td>
</tr>
<tr>
<td>Chr1 Sat2 hypomethylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2 vs. ≤2</td>
<td>4.1 (1.2–14.7)</td>
<td>0.029</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60 yr vs. ≤60 yr</td>
<td>7.2 (1.5–34.3)</td>
<td>0.014</td>
</tr>
<tr>
<td>Figo stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III/IV vs. I/II</td>
<td>0.2 (0.1–1.0)</td>
<td>0.049</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III vs. I/II</td>
<td>0.3 (0.1–1.8)</td>
<td>0.19</td>
</tr>
<tr>
<td>Chr1 Sat2 hypomethylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;2 vs. ≤2</td>
<td>9.4 (2.1–41.5)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<sup>a</sup> LMP, low malignant potential; FIGO, Fédération Internationale des Gynaecologistes et Obstetristes; CI, confidence interval.

cell carcinoma of the lung (33), can be an independent prognostic indicator.

How could satellite DNA hypomethylation influence ovarian cancer? It might be of biological significance by itself, or it may be an indication only of DNA methylation changes at oncogenically relevant targets elsewhere in the genome. Because cancer-associated satellite DNA demethylation might be an inducer of de novo methylation of transcription control regions of tumor suppressor genes, we tested methylation of CpG islands at the 5' ends of 15 genes, many of which are hypermethylated in certain cancers. Only 1 of these, CALCA, showed a significant positive association between its hypermethylation and hypomethylation of satellite DNA, and this gene is not implicated in carcinogenesis. Rather, it appears to be an epigenetic marker of cancer only (34). Therefore, as we had shown for Wilms' tumors (30), it is unlikely that cancer-linked satellite DNA hypomethylation acts only as an inducer of or responder to cancer-linked hypermethylation in multiple gene regions. We did demonstrate an inverse correlation between satellite DNA hypomethylation and hypermethylation of CDH13, which encodes a cadherin suspected to be a tumor suppressor gene (35, 36). Such an inverse correlation was seen also for RNR1, which encodes rRNA and of which the hypomethylation has been reported in breast cancer (37). For example, only 23% of all of the ovarian carcinomas in this study did not exhibit any Chr1 Sat2 hypomethylation, but among the 12 carcinomas with much hypermethylation of CDH13 (percentage of fully methylated reference >10), 67% showed no Chr1 Sat2 hypomethylation. These observations suggest an antagonistic relationship between satellite DNA hypomethylation and hypermethylation of a small subset of genes subject to cancer-associated DNA hypermethylation. Our findings indicate also that CDH13 or RNR1 hypermethylation might serve as a surrogate marker for satellite DNA hypomethylation.

Attention has been focused recently on the role of DNA hypomethylation in chromosome instability (19, 38–43). Genomic instability and DNA hypomethylation are observed often early during carcinogenesis (15, 44). Moreover, gross chromosomal changes and point mutagenesis typically increase with tumor progression. Here, we have demonstrated for ovarian carcinomas that hypomethylation of satellite DNA at 1qh and in the adjacent centromere is significantly more prevalent with tumor progression in ovarian cancers. Centromeres and the 1qh region often display unbalanced rearrangements (45) that could contribute to carcinogenesis by the resulting gene imbalances. There are several types of evidence relating satellite DNA hypomethylation or general DNA hypomethylation to genomic instability. In patients with the immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome, a relationship was found between naturally occurring Sat2 hypomethylation at 1qh and 16qh and frequent pericentromeric rearrangements at or adjacent to these chromosomal bands in lymphoid cells (42, 46). Although ICF patients display no increased cancer incidence, ~50 patients (mostly children) have been identified, and their very short average life span would preclude detection of a cancer predisposition that was not very high and did not result in tumors rather quickly. An experimental link between DNA demethylation and chromosome instability was seen in studies demonstrating that the demethylating agents 5-azacytidine and 5-azadecoxycytidine induce high levels of pericentromeric rearrangements specifically targeted at or adjacent to 1qh and 16qh in normal lymphoid cells (47, 48).

Although hypomethylation of pericentromeric DNA in heterochro-
DNA HYPMETHYLATION IN OVARIAN CANCER

Table 4 Association of CpG island hypermethylation in 5′ gene regions with satellite DNA hypomethylation

A. Association of satellite DNA hypomethylation and gene hypermethylation in ovarian cancers

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RNR1</td>
<td>–</td>
<td>&lt;0.001</td>
<td>CDH13</td>
<td>–</td>
<td>&lt;0.001</td>
<td>CDH13</td>
<td>–</td>
<td>0.014</td>
</tr>
<tr>
<td>CDH13</td>
<td>–</td>
<td>0.001</td>
<td>RNR1</td>
<td>+</td>
<td>0.003</td>
<td>CALCA</td>
<td>+</td>
<td>0.13</td>
</tr>
<tr>
<td>CALCA</td>
<td>–</td>
<td>0.11</td>
<td>ESR1</td>
<td>–</td>
<td>0.24</td>
<td>APc</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>ESR1</td>
<td>+</td>
<td>0.31</td>
<td>APc</td>
<td>+</td>
<td>0.09</td>
<td>MCJ</td>
<td>+</td>
<td>0.51</td>
</tr>
<tr>
<td>APc</td>
<td>+</td>
<td>0.55</td>
<td>MCJ</td>
<td>+</td>
<td>0.40</td>
<td>TERT</td>
<td>–</td>
<td>0.54</td>
</tr>
<tr>
<td>BLT1</td>
<td>+</td>
<td>0.55</td>
<td>SOCS1</td>
<td>–</td>
<td>0.55</td>
<td>BLT1</td>
<td>+</td>
<td>0.55</td>
</tr>
<tr>
<td>CDH1</td>
<td>+</td>
<td>0.60</td>
<td>PTGS2</td>
<td>+</td>
<td>0.55</td>
<td>DR3</td>
<td>–</td>
<td>0.71</td>
</tr>
<tr>
<td>PTGS2</td>
<td>+</td>
<td>0.70</td>
<td>APc</td>
<td>+</td>
<td>0.61</td>
<td>PTGS2</td>
<td>+</td>
<td>0.71</td>
</tr>
<tr>
<td>MCJ</td>
<td>+</td>
<td>0.78</td>
<td>PGR</td>
<td>–</td>
<td>0.68</td>
<td>IGSF4</td>
<td>–</td>
<td>0.72</td>
</tr>
<tr>
<td>TNFRSF12</td>
<td>–</td>
<td>0.91</td>
<td>IGSF4</td>
<td>–</td>
<td>0.73</td>
<td>PGR</td>
<td>–</td>
<td>0.86</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>–</td>
<td>0.92</td>
<td>DR3</td>
<td>+</td>
<td>0.79</td>
<td>RASSF1A</td>
<td>+</td>
<td>0.90</td>
</tr>
<tr>
<td>TERT</td>
<td>–</td>
<td>0.94</td>
<td>CALCA</td>
<td>+</td>
<td>0.83</td>
<td>ESR1</td>
<td>+</td>
<td>1.0</td>
</tr>
</tbody>
</table>

B. Assocn. of satellite hypomethylation with gene hypermethylation in breast cancers

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH13</td>
<td>–</td>
<td>0.030</td>
<td>RNR1</td>
<td>–</td>
<td>0.15</td>
<td>CALCA</td>
<td>–</td>
<td>0.49</td>
</tr>
<tr>
<td>RNR1</td>
<td>–</td>
<td>0.10</td>
<td>CALCA</td>
<td>–</td>
<td>0.12</td>
<td>–</td>
<td>0.030</td>
<td>–</td>
</tr>
<tr>
<td>CALCA</td>
<td>–</td>
<td>0.84</td>
<td>–</td>
<td>–</td>
<td>0.055</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* By the Mann-Whitney U test, the association of 15 gene hypermethylation markers in ovarian cancer specimens are ranked according to the strength of their association with three different categories of satellite DNA hypomethylation (Sat2, juxta-centromeric Sat2 in Chr1; Chr1 SatO, centromeric SatO in Chr1; All centromeres, all centromeric SatO DNA). The satellite DNA hypomethylation scores were categorized as to those ≤2 versus those >2. The PMR values of the 15 genes were used as continuous variables. Significant associations after adjustment for multiple comparisons are indicated in bold type.

* Assocn. +, a positive relationship (not necessary significant) between the extent of satellite DNA hypomethylation and DNA hypermethylation; Assocn. −, tumors with hypermethylation of this gene marker had a lower frequency of satellite DNA hypomethylation than seen for the complete collection of tumors.

matin may predispose certain human cell populations to rearrangements in these regions, e.g., ICF lymphoid cells and hepatocellular carcinoma (49), as does hypomethylation of euchromatic DNA sequences elsewhere (41, 50), exceptions to the association of Sat2 DNA hypomethylation with pericentromeric rearrangements have been reported for breast carcinomas (51). Moreover, our recent study of Wilms’ tumors involving a detailed karyotype analysis and examination of satellite DNA methylation showed that the frequencies of hypomethylation at BstBI sites in Chr1 Sat2 and at SatO throughout the centromeres (51% and 69% of 35 primary tumors, respectively, compared with various normal postnatal somatic tissues) were much greater than the frequencies of pericentromeric rearrangements in Chr1 or in any of the chromosomes (14 and 20%, respectively; Ref. 19). Similarly, the very high frequencies of cancer-associated hypomethylation at Chr1 Sat2 and at SatO throughout the centromeres seen in the present study (52% and 51%, respectively, compared with normal ovaries, or 77% and 99%, compared with various other normal postnatal somatic tissues) suggest that the functional significance of this hypomethylation is not limited to fostering chromosome rearrangements.

Other possible roles of DNA hypomethylation in cancer relate to either cis- or, possibly, trans-effects on gene expression. Because satellite DNA hypomethylation in ovarian carcinomas, Wilms’ tumors, and breast adenocarcinomas has been shown to be significantly associated with global DNA hypomethylation (21, 30), there may be waves of DNA hypomethylation that typically include satellite DNA sequences but involve gene targets also that impact tumor formation and progression. Satellite DNA hypomethylation might spread additionally to adjacent euchromatin regions. Although it does not seem that activation of DNA methylation-repressed retrotransposons plays a major role in cancers (39), there is growing evidence that some (52–55), but not all (17), of the gene targets of cancer-associated demethylation may get turned on by this hypomethylation and contribute to carcinogenesis. Furthermore, there is a heightened appreciation of the importance of intranuclear localization of chromosomal regions in the regulation of expression of certain genes (56). Evidence indicates that centromeric heterochromatin can interact in trans with genes dispersed in the genome to help control their expression. This might be mediated by different types of constitutive heterochromatin serving as reservoirs for specific DNA-binding proteins (57).

Whatever the most important biological target of cancer-associated genomic hypomethylation, it should be noted that decreases in DNA methylation induced as part of a therapeutic regimen might contribute to carcinogenesis (15, 38, 39) or tumor progression (32). Attempts to decrease DNA methylation in neoplasias as a therapeutic strategy by using 5-azacytidine or 5-aza-2′-deoxycytidine have been productive in hematological malignancies but disappointing in solid tumors (58). Azacytidine has been shown to enhance the formation of lung tumors (59) in mice, testicular and liver cancer (60) in rats, and to have oncogenic effects on cultured cells (61). A Phase II study of 5-aza-2′-deoxycytidine in patients with advanced ovarian carcinoma showed no activity (62). Our finding that an increase in DNA hypomethylation is associated with an increase in aggressiveness of ovarian cancers and with a decrease in patient survival calls for caution in using demethylating agents as an anticancer drug.

REFERENCES


* M. Ehrlich, K. Jackson, E. Fiala, and M. Widschwendter, unpublished observations.
DNA HYPMETHYLATION IN OVARIAN CANCER


DNA Hypomethylation and Ovarian Cancer Biology

Martin Widschwendter, Guanchao Jiang, Christian Woods, et al.


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