[CANCER RESEARCH 64, 4472–4480, July 1, 2004]

DNA Hypomethylation and Ovarian Cancer Biology

Martin Widschwendter,[1] Guanchao Jiang,[3] Christian Woods,[3] Hannes M. Müller,[1] Heidi Fiegl,[1] Georg Goebel,[2] Christian Marth,[1] Elisabeth Müller-Holzner,[1] Alain G. Zeimet,[1] Peter W. Laird,[4] and Melanie Ehrlich[3]

Departments of [1]Obstetrics and Gynecology and [2]Biostatistics and Documentation, Medical University Innsbruck, Innsbruck, Austria; [3]Tulane Cancer Center, Human Genetics Program, and Department of Biochemistry, Tulane Medical School, New Orleans, Louisiana; and Departments of [4]Surgery, Biochemistry, and Molecular Biology, University of Southern California, Norris Comprehensive Cancer Center, Los Angeles, California

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 5′ 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 *RNR1* (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 genomic 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

Received 1/23/04; revised 3/29/04; accepted 4/19/04.

Grant support: Grants from the Fonds zur Förderung der wissenschaftlichen Forschung, P15995-B05 and P16159-B05 (M. Widschwendter), the Austrian National Bank #9856 (M. Widschwendter), NIH Grants R01-CA81506 (M. Ehrlich) and R01-CA096958 (P. Laird).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

Requests for reprints: Melanie Ehrlich, Human Genetics Program SL31, Tulane Medical School, New Orleans, LA 70112. Phone: (504) 584-2449; Fax: (504) 584-1763; E-mail: ehrlich@tulane.edu; and Martin Widschwendter, Department of Obstetrics and Gynecology, Innsbruck University Hospital, Anichstr. 35, A-6020 Innsbruck, Austria. Phone: 0043 512 504 4155; Fax: 0043 512 504 3112; E-mail: martin.widschwendter@uibk.ac.at.

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 lyophilization. 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 QIAmp tissue kit (Qiagen, Hilden, Germany). Southern blot analysis with a CpG methylation-sensitive restriction endonuclease, *Bst*BI, was done to assess the extent of hypomethylation in satellite DNA (0.5 μg) under high-stringency (Chr1 Sat2 and Chr1 Satα) or low-stringency (Satα in the centromeres throughout the genome) conditions with a 1.77-kb Chr1 Sat2 (23) or a 1.9-kb Chr1 Satα (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 Chr1 Sat2 and Chr1 Satα hypomethylation, we used two criteria. The first was phosphorimager 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. Chr1 Sat2 hypomethylation scores for tumors, in italics, followed by typical R values were as follows: *0,* <1.8; *1,* 1.8–2.5; *2,* 2.6–5.0; *3,* 5.1–8.0; and *4,* >8.0 compared with sperm with a R value of ~25. For Chr1 Satα 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 Satα 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 Satα sequence, including at the *Bst*BI 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 Chr1 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 digests with CpG methylation-insensitive enzymes and a moderately repeated DNA sequence (D4Z4) as a blot hybridization probe. Comparison of the ratios of the phosphorimager 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*, CGTTTTGGAGATACGGGTCG, AAACAACGCCGAACCGAA, 6FAM-ACCGCCCGTACCACACGCAAA-BHQ-1; *MCJ*, TTTCGGGTCGTTTTGTTATGG, ACTACAAATACTCAACGTAACGCAAACT, 6FAM-TCGCCAACTAAAACGATAACACCACGAACA-BHQ-1; *TNFRSF12*, GCGGAATTACGACGGGTAGA, ACTCCATAACCCTCCGACGA, 6FAM-CGCCCAAAAACTTCCCGACTCCGTA-BHQ-1; and *IGSF4*, GGGTTTCGGAGGTAGTTAACGTC, CACTAAAATCCGCTCGACAACAC, 6FAM-ACACTCGCCATATCGAACACCTACCTCAAA-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. Associations of hypomethylation and clinicopathological features were determined using the χ^2 contingency test and Spearman rank coefficient. Associations of dichotomized hypomethylation values and hypermethylation measures of 15 different genes are expressed as rankings using Mann-Whitney *U* test statistics and the Benjamini-Hochberg multiple test adjustment (29). For univariate survival analysis, we used Kaplan-Meier curves and log-rank test statistics. Multivariate survival analysis was done using a time-independent proportional hazard model adjusted for age, International Federation of Gynecology and Obstetrics, and tumor stage. All of the statistical calculations were performed using SPSS, version 10.0.

RESULTS

DNA Hypomethylation Status in Non-Neoplastic *versus* Neoplastic Ovarian Tissue Specimens. Ovarian cancers (115 specimens) from previously untreated patients and non-neoplastic ovarian tissue (26 specimens) from noncancer patients were analyzed for the extent of satellite DNA hypomethylation. Methylation was examined in juxtacentromeric and centromeric DNAs (Sat2 and Satα, respectively) in Chr1 and in centromeric Satα throughout the genome by Southern blot analysis of *Bst*BI digests. Hypomethylation of ovarian samples was quantitated approximately by comparison to normal postnatal somatic tissues, which are all highly methylated in these satellite sequences. This analysis was done in a blinded fashion with respect to the clinicopathological features of the ovarian samples (Table 1), and assessment of satellite DNA hypomethylation was by quantitative phosphorimager analysis as well as by visually comparing banding patterns. We had shown that satellite DNA hypomethylation analyzed in this manner is correlated significantly with global 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 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 Chr1 Sat 2, Chr1 Satα, and Satα 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 Chr1 Sat2, and only 4% (1 sample) had a hypomethylation score >1 for Chr1 Satα or Satα throughout the centromeres (Table 1). However, 12, 43, and 84% of the non-neoplastic ovarian specimens (most of which were normal,

Table 1 *Clinicopathological features and DNA hypomethylation analysis of ovarian cancers and non-neoplastic ovaries*

DNA hypomethylation levels have been numbered 0 to 4. Ovarian cancer cases have been ranked with the increase in stage and grade.

Age[a]	Cancer	FIGO	Histo	MG	RemTu	Sat2	Satα	All c
33	no		CYS			0	1	1
81	no		OVA			0	ND	1
67	no		OVA			0	0	0
62	no		OVA			0	0	1
80	no		OVA			0	ND	1
69	no		OVA			0	0	1
31	no		CYS			0	1	ND
72	no		OVA			0	0	0
34	no		OVA			0	1	1
66	no		OVA			0	ND	1
74	no		CYS			0	1	0
30	no		OVA			0	0	1
75	no		OVA			0	0	1
62	no		OVA			0	0	1
65	no		OVA			0	1	1
66	no		OVA			0	0	1
38	no		CYS			0	0	1
86	no		OVA			0	1	1
60	no		OVA			0	1	1
61	no		OVA			1	0	1
73	no		OVA			0	0	1
31	no		CYS			0	1	1
26	no		CYS			0	1	1
27	no		CYS			1	1	1
71	no		OVA			0	0	1
59	no		OVA			1	2	2
71	yes	NA	SER	II	NA	2	3	1
75	yes	I	MUC	I	no	0	1	1
70	yes	I	SER	I	no	1	2	2
66	yes	I	LMP	I	no	0	ND	1
72	yes	I	LMP	I	no	0	0	1
22	yes	I	LMP	I	no	0	1	1
39	yes	I	LMP	I	no	0	1	1
50	yes	I	LMP	I	no	1	2	1
23	yes	I	LMP	I	no	1	1	2
83	yes	I	LMP	I	no	2	2	2
56	yes	I	END	II	no	1	2	ND
52	yes	I	MUC	II	no	0	ND	1
48	yes	I	MUC	II	no	1	0	1
58	yes	I	SER	II	no	0	1	1
49	yes	I	MUC	II	no	0	1	1
83	yes	I	SER	II	no	0	2	1
72	yes	I	MUC	II	no	0	2	1
44	yes	I	MUC	II	no	1	2	1
63	yes	I	END	II	no	0	1	2
79	yes	I	MUC	II	no	1	1	2
87	yes	I	MUC	II	no	1	1	2
53	yes	I	SER	II	no	1	2	2
51	yes	I	MUC	II	no	1	2	2
36	yes	I	MUC	II	no	2	2	2
48	yes	I	END	II	no	2	2	2
50	yes	I	MUC	II	no	3	1	1
70	yes	I	MUC	II	no	3	2	2
75	yes	I	END	II	no	3	2	2
63	yes	I	MUC	III	no	1	2	1
78	yes	I	SER	III	no	4	2	1
43	yes	II	LMP	I	no	0	1	1
83	yes	II	MUC	II	yes	1	1	1
40	yes	II	MUC	II	yes	0	2	2
49	yes	II	END	III	no	2	2	2
69	yes	II	SER	III	no	3	2	1
64	yes	II	SER	III	yes	3	2	1
81	yes	II	SER	III	no	2	3	3
44	yes	II	END	III	no	2	4	3
64	yes	III	MUC	II	no	1	2	ND
63	yes	III	SER	II	no	0	1	1
79	yes	III	SER	II	no	0	1	1
67	yes	III	SER	II	yes	1	1	1
66	yes	III	MUC	II	yes	1	1	1
51	yes	III	END	II	no	2	1	1
50	yes	III	SER	II	yes	2	1	1
74	yes	III	SER	II	yes	0	2	1
68	yes	III	MUC	II	yes	0	2	1
74	yes	III	MUC	II	no	1	2	1
42	yes	III	MUC	II	no	2	2	1
56	yes	III	END	II	no	2	2	1
79	yes	III	SER	II	NA	2	2	1
66	yes	III	MUC	II	no	0	1	2
57	yes	III	MUC	II	yes	0	2	2
53	yes	III	SER	II	no	1	2	2
36	yes	III	MUC	II	no	1	2	2 .

Table 1 *Continued.*

Age[a]	Cancer	FIGO	Histo	MG	RemTu	Sat2	Satα	All c
74	yes	III	SER	II	yes	1	2	2
65	yes	III	MUC	II	yes	1	2	2
78	yes	III	SER	II	yes	1	2	2
25	yes	III	MUC	II	yes	2	2	2
77	yes	III	SER	II	yes	3	2	0
63	yes	III	SER	II	yes	3	1	1
59	yes	III	SER	II	yes	2	3	1
24	yes	III	SER	II	yes	2	3	2
67	yes	III	MUC	II	yes	2	3	2
58	yes	III	SER	II	yes	2	3	2
58	yes	III	SER	II	no	3	3	1
42	yes	III	SER	II	yes	3	3	1
62	yes	III	MUC	II	no	4	3	1
69	yes	III	MUC	II	yes	4	3	2
61	yes	III	SER	II	yes	4	3	2
52	yes	III	SER	II	yes	3	4	2
33	yes	III	MUC	II	yes	4	4	2
63	yes	III	SER	II	no	2	3	3
72	yes	III	SER	II	yes	3	4	3
73	yes	III	MUC	II	no	4	4	3
81	yes	III	END	II	no	4	4	3
49	yes	III	END	II	no	4	4	3
82	yes	III	MUC	II	yes	4	4	3
64	yes	III	SER	II	yes	4	4	3
50	yes	III	MUC	II	yes	4	4	4
76	yes	III	MUC	II	yes	0	1	1
55	yes	III	MUC	III	yes	0	1	1
72	yes	III	MUC	III	NA	0	1	1
77	yes	III	SER	III	yes	1	2	1
81	yes	III	MUC	III	yes	2	2	1
48	yes	III	MUC	III	yes	2	2	1
71	yes	III	SER	III	yes	0	1	2
55	yes	III	MUC	III	yes	0	1	2
56	yes	III	END	III	yes	1	1	2
46	yes	III	SER	III	NA	1	2	2
61	yes	III	MUC	III	yes	2	2	2
57	yes	III	SER	III	yes	2	2	2
77	yes	III	SER	III	yes	3	1	1
55	yes	III	MUC	III	yes	1	3	1
60	yes	III	SER	III	NA	2	3	1
67	yes	III	SER	III	yes	2	3	2
58	yes	III	END	III	yes	4	3	2
44	yes	III	SER	III	yes	4	4	2
50	yes	III	SER	III	yes	2	4	4
73	yes	III	END	III	no	4	4	3
50	yes	III	SER	III	yes	4	4	3
73	yes	III	SER	III	yes	4	4	3
71	yes	III	SER	III	yes	4	4	4
79	yes	III	SER	III	yes	4	4	4
64	yes	IV	END	II	yes	1	ND	1
73	yes	IV	MUC	II	yes	0	0	1
48	yes	IV	SER	II	yes	1	0	1
66	yes	IV	SER	II	yes	3	0	1
76	yes	IV	MUC	II	yes	3	4	3
73	yes	IV	END	III	yes	0	ND	ND
47	yes	IV	SER	III	no	0	1	1
60	yes	IV	SER	III	yes	1	2	2
74	yes	IV	SER	III	yes	3	3	1
57	yes	IV	SER	III	no	4	3	1
57	yes	IV	END	III	yes	3	3	2

[a] Age, age of the corresponding patient in years; Cancer, yes if ovarian cancer and no if non-neoplastic ovary; FIGO, Fédération Internationale des Gynaecologistes et Obstetristes 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, Satα, All c, DNA hypomethylation levels at Chr1 Sat2, Chr1 Satα, and Satα throughout the centromeres, respectively, on a scale of no hypomethylation (0) to maximum observed hypomethylation (4), as illustrated in Fig. 1

whole ovaries) displayed a slight amount of Chr1 Sat 2, Chr1 Satα, or general Satα 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 >2 (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 >2 for Chr1 Sat 2, Chr1 Satα, or Satα 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

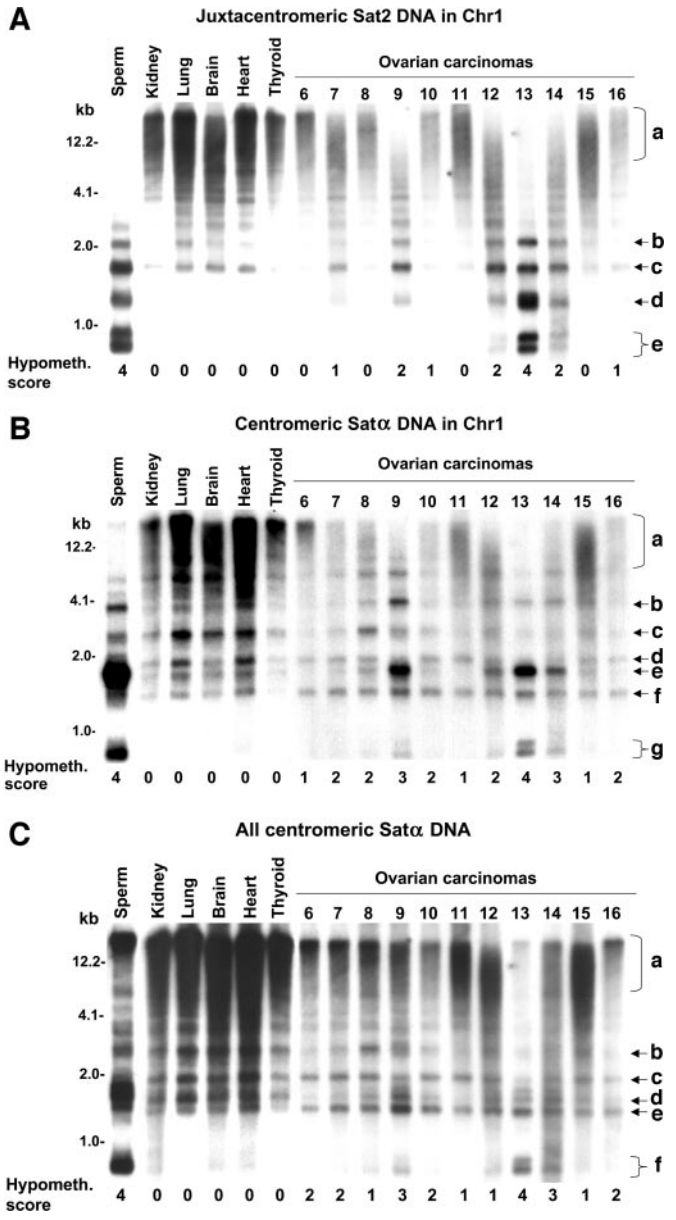

Fig. 1. Hypomethylation of satellite DNA in ovarian adenocarcinomas. A representative blot showing different amounts of hypomethylation of satellite DNA in *Bst*BI digests of ovarian carcinomas in a single blot hybridized three times with stripping in between. Normal postnatal somatic DNAs and sperm DNA are the hypermethylated and hypomethylated standards, respectively. *A,* juxtacentromeric Sat2 probe from Chr1 used under high-stringency hybridization conditions. *B,* centromeric Satα from Chr1 used under high-stringency hybridization conditions. *C,* centromeric Satα from Chr1 used under low-stringency conditions that allow hybridization to DNA from most of the centromeres. Scoring of hypomethylation levels (from 0–4) involved phosphorimager analysis of relative amounts of the total low-molecular-weight (<4-kb) and total high-molecular-weight (>4-kb) hybridizing signal, and changes in band intensities as follows. *A,* relative to region *a*: hypomethylation score 1, increases in *b* or *c*; 2, increases in *d* or *e*; and 3, increases in *c*–*e*. *B,* relative to region *a* or band *c*: hypomethylation score 1, increases in *d*, *e*, or *f*; 2, increases in *e* or larger increases in *f*; and 3, larger increases in *e*. *C,* relative to band *b*: hypomethylation score 1, increases in *e* and usually *c*; 2, additional increases in *e*; and 3, large increases in *d* or *e*. For all of the panels, samples designated as having a hypomethylation score of 4 are very similar in profile to sperm DNA, the hypomethylated reference.

the age of the patients (≤60 *versus* >60 years) and the extent of satellite hypomethylation (Table 2). Advanced stage and high tumor grade were associated significantly with much hypomethylation of Chr1 Sat2 or Chr1 Satα. Serous and endometrioid ovarian cancers demonstrated significantly higher DNA hypomethylation levels than did LMP or mucinous tumors. Also, tumors from patients who did not get optimal cytoreductive surgery had significantly more hypomethylation of Chr1 Satα (Table 2). This finding might be related to the observation that patients who do not undergo this type of surgery often have a larger tumor burden.

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 (neither 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). However, patients whose tumors demonstrated no hypomethylation of Chr1 Sat2 or Chr1 Satα or only lower levels of this hypomethylation (0, 1, or 2 on a scale of 0–4) had a significantly better relapse-free survival compared with patients with strong hypomethylation at these regions (hypomethylation scores of 3 or 4; Fig. 2). The level of Chr1 Sat2 hypomethylation was a prognostic marker with regard to overall survival as well as relapse-free survival. To assess whether 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 3*A*) and death was 9.4 (Table 3*B*).

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 Satα, or Satα throughout the centromeres are shown in Table 4*A*. Hypermethylation of *CDH13*, a cadherin family gene at 16q24, 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 Satα throughout the centromeres, whereas the same trend was seen for *RNR1* and the examined satellite DNAs (Table 4*B*; 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

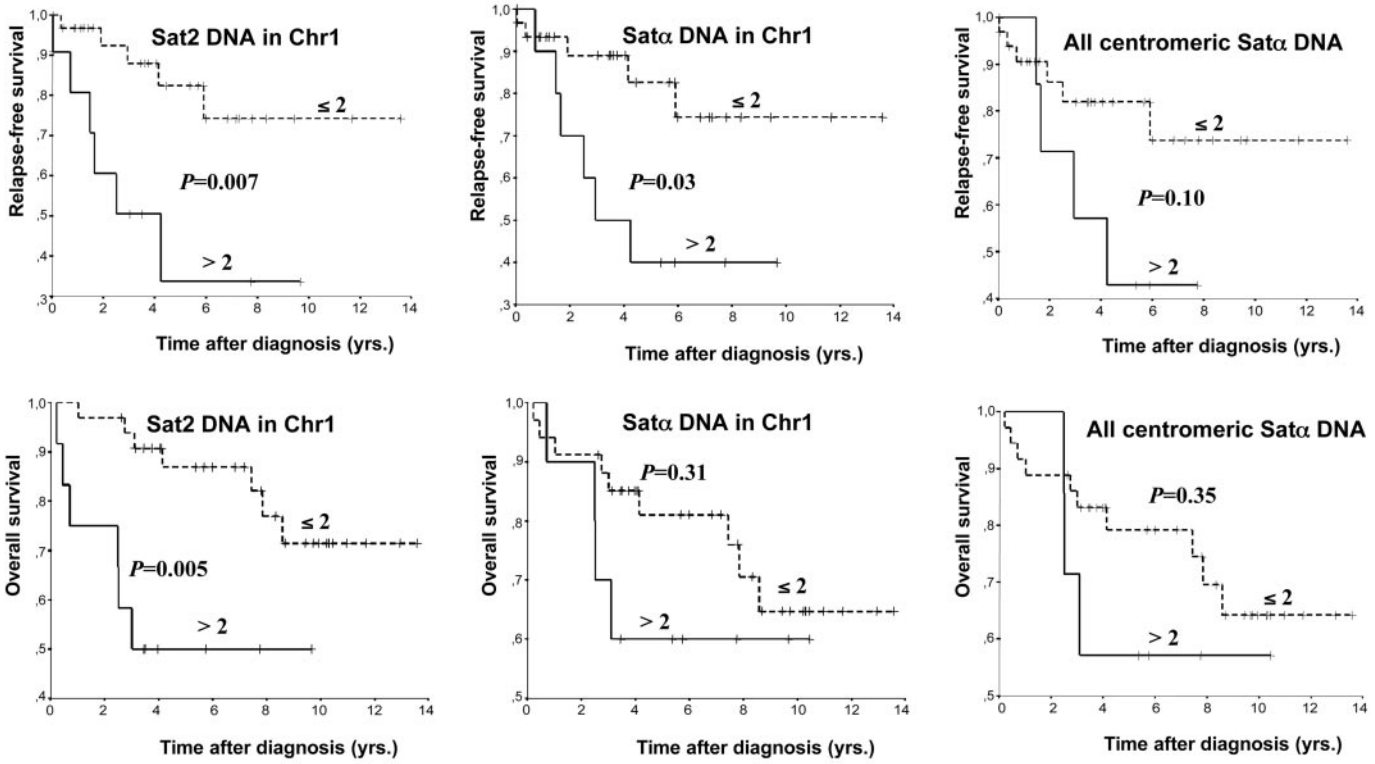

Fig. 2. Kaplan-Meier analysis of the probability of relapse-free as well as overall survival among all of the 45 non-LMP ovarian cancer patients with optimal surgical cytoreductive therapy. Samples with satellite hypomethylation scores of ≤2 had no, slight, or only moderate hypomethylation, whereas those with scores of >2 had much hypomethylation. Log-rank *P*s are shown.

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, *Bst*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 Satα in Chr1, and Satα 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

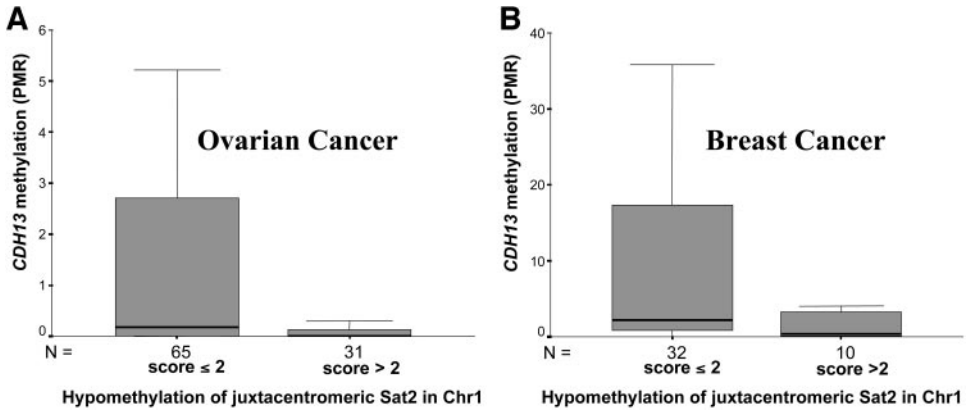

Fig. 3. Association of Chr1 Sat2 hypomethylation and *CDH13* hypermethylation in ovarian and breast cancer specimens. A box plot is shown with the *thick horizontal line* depicting the median PMR value, the *box* highlighting the 25th to 75th percentile, and the *brackets* indicating the maximum and minimum values excluding outliers. Chr1 Sat2 hypomethylation scores of >2 (on a scale of 0–4) indicate strong hypomethylation at this heterochromatin region. High PMR values, determined by the MethyLight assay, refer to much hypermethylation at *CDH13* assayed at positions +131 to +233 relative to the transcription start site. Outliers have been omitted from this figure but were included in the rank-based statistics seen in Table 4; *bars*, ±SD.

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

	Satellite DNA hypomethylation score								
	Chr1 Sat2[a]			Chr1 Satα[a]			All centromeres[a]		
Variable	≤2 (n = 80)	>2 (n = 35)	*P*	≤2 (n = 74)	>2 (n = 37)	*P*	≤2 (n = 95)	>2 (n = 17)	*P*
Age									
≤60 yr	40	12		33	18		46	5	
>60 yr	40	23	0.16	41	19	0.69	49	12	0.19
Stage[b]									
FIGO[c] I/II	31	6		33	2		34	2	
FIGO III/IV	48	29	0.029	41	34	<0.001	60	15	0.053
Grading									
MG I	10	0		9	0		10	0	
MG II	48	21		46	21		58	9	
MG III	22	14	0.038	19	16	0.012	27	8	0.063
Histology									
LMP	8	0		7	0		8	0	
Serous	29	20		27	22		40	9	
Mucinous	33	9		32	9		37	4	
Endometrioid	10	6	0.046	8	6	0.024	10	4	0.20
Remaining tumor[b]									
No	41	12		41	10		44	7	
Yes	34	23	0.065	30	25	0.007	46	10	0.61

[a] 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.
[b] Information about stage and remaining tumor is missing in 1 and 5 cases, respectively.
[c] FIGO, Fédération Internationale des Gynaecologistes et Obstetristes; LMP, low malignant potential.

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 hypermethylation 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.

Table 3 *Multivariate analysis for relapse-free and overall survival of the 45 non-LMP[a] ovarian cancer cases with optimal surgical cytoreductive therapy*

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

[a] LMP, low malignant potential; FIGO, Fédération Internationale des Gynaecologistes et Obstetristes; CI, confidence interval.

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-azadeoxycytidine 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-

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

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

Chr1 Sat2			Chr1 Satα			All centromeres		
Genes	Assocn.[b]	*P*	Genes	Assocn.	*P*	Genes	Assocn.	*P*
RNR1	−	**<0.001**	CDH13	−	**<0.001**	CDH13	−	**0.014**
CDH13	−	**0.001**	BLT1	−	0.03	CALCA	+	0.13
CALCA	+	**0.003**	RNR1	−	0.09	RNR1	−	0.20
ESR1	−	0.11	MCJ	−	0.09	MCJ	−	0.36
APC	+	0.24	RASSF1A	+	0.11	SOCS1	+	0.43
BLT1	+	0.42	ESR1	+	0.14	CDH1	+	0.51
CDH1	+	0.53	CDH1	+	0.31	APC	+	0.52
PGR	−	0.55	SOCS1	−	0.49	TERT	+	0.54
SOCS1	−	0.60	PTGS2	−	0.55	BLT1	+	0.55
PTGS2	+	0.60	TERT	−	0.55	DR3	−	0.71
MCJ	+	0.70	APC	−	0.61	PTGS2	+	0.71
TNFRSF12	−	0.78	PGR	−	0.68	IGSF4	+	0.72
RASSF1A	−	0.91	IGSF4	−	0.73	PGR	−	0.86
TERT	−	0.92	DR3	+	0.79	RASSF1A	−	0.90
IGSF4	+	0.94	CALCA	+	0.83	ESR1	+	1.0

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

	Chr1 Sat2		Chr1 Satα		All centromeres	
Genes	Assocn.	*P*	Assocn.	*P*	Assocn.	*P*
CDH13	−	**0.030**	−	0.10	−	**0.030**
RNR1	−	0.15	−	0.12	−	**0.030**
CALCA	−	0.49	−	0.84	−	0.055

[a] 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, juxtacentromeric Sat2 in Chr1; Chr1 Satα, centromeric Satα in Chr1; All centromeres, all centromeric Satα 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.

[b] 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 *Bst*BI sites in Chr1 Sat2 and at Satα 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 Satα 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),[5] 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.

[5] M. Ehrlich, K. Jackson, E. Fiala, and M. Widschwendter, unpublished observations.

REFERENCES

1. Jemal A, Murray T, Samuels A, et al. Cancer statistics, 2003. CA - Cancer J Clin 2003;53:5–26.
2. Holschneider CH, Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. Semin Surg Oncol 2000;19:3–10.
3. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics, 1999. CA - Cancer J Clin 1999;49:8–31.
4. Aunoble B, Sanches R, Didier E, Bignon YJ. Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer [review]. Int J Oncol 2000;16:567–76.
5. Suzuki S, Moore DH, Ginzinger DG, et al. An approach to analysis of large-scale correlations between genome changes and clinical endpoints in ovarian cancer. Cancer Res 2000;60:5382–5.

6. Ono K, Tanaka T, Tsunoda T, et al. Identification by cDNA microarray of genes involved in ovarian carcinogenesis. Cancer Res 2000;60:5007–11.
7. Welsh JB, Zarrinkar PP, Sapinoso LM, et al. Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. Proc Natl Acad Sci USA 2001;98:1176–81.
8. Chan KY, Ozcelik H, Cheung AN, Ngan HY, Khoo US. Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. Cancer Res 2002;62: 4151–6.
9. Yoon JH, Dammann R, Pfeifer GP. Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. Int J Cancer 2001;94:212–7.
10. Ahluwalia A, Hurteau JA, Bigsby RM, Nephew KP. DNA methylation in ovarian cancer. II. Expression of DNA methyltransferases in ovarian cancer cell lines and normal ovarian epithelial cells. Gynecol Oncol 2001;82:299–304.
11. Ahluwalia A, Yan P, Hurteau JA, et al. DNA methylation and ovarian cancer. I. Analysis of CpG island hypermethylation in human ovarian cancer using differential methylation hybridization. Gynecol Oncol 2001;82:261–8.
12. Strathdee G, Appleton K, Illand M, et al. Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. Am J Pathol 2001; 158:1121–7.
13. Feinberg AP. Cancer epigenetics takes center stage. Proc Natl Acad Sci USA 2001;98:392–4.
14. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
15. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002;21:5400–13.
16. Muller HM, Widschwendter M. Methylated DNA as a possible screening marker for neoplastic disease in several body fluids. Expert Rev Mol Diagn 2003;3:443–48.
17. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature (Lond) 1983;301:89–92.
18. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res 1983;11:6883–94.
19. Ehrlich M, Hopkins NE, Jiang G, et al. Satellite DNA hypomethylation in karyotyped Wilms' tumors. Cancer Genet Cytogenet 2003;141:97–105.
20. Narayan A, Ji W, Zhang XY, et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 1998;77:833–8.
21. Qu G, Dubeau L, Narayan A, Yu MC, Ehrlich M. Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. Mutat Res 1999;423:91–101.
22. Cheng P, Schmutte C, Cofer KF, et al. Alterations in DNA methylation are early, but not initial, events in ovarian tumorigenesis. Br J Cancer 1997;75:396–402.
23. Cooke H. Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 1979;10:3177–97.
24. Waye J, Durfy S, Pinkel D, et al. Chromosome-specific alpha satellite DNA from human chromosome 1: hierarchical structure and genomic organization of a polymorphic domain spanning several hundred kilobase pairs of centromeric DNA. Genomics 1987;1:43–51.
25. Eads CA, Lord RV, Kurumboor SK, et al. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res 2000;60:5021–6.
26. Eads CA, Lord RV, Wickramasinghe K, et al. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res 2001;61:3410–8.
27. Eads CA, Danenberg KD, Kawakami K, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.
28. Muller HM, Widschwendter A, Fiegl H, et al. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res 2003;63:7641–5.
29. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001;125:279–84.
30. Ehrlich M, Jiang G, Fiala E, et al. Hypomethylation and hypermethylation of DNA in Wilms' tumors. Oncogene 2002;21:6694–702.
31. Paz MF, Avila S, Fraga MF, et al. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res 2002;62:4519–24.
32. Itano O, Ueda M, Kikuchi K, et al. Correlation of postoperative recurrence in hepatocellular carcinoma with demethylation of repetitive sequences. Oncogene 2002;21:789–97.
33. Brabender J, Usadel H, Danenberg KD, et al. Adenomatous polyposis coli gene promoter hypermethylation in non-small cell lung cancer is associated with survival. Oncogene 2001;20:3528–32.
34. Issa JP. Hypermethylator phenotypes in aging and cancer. In Ehrlich M, editor. Natick, Massachusetts: Eaton Publishing; 2000. p. 311–22.
35. Roman-Gomez J, Castillejo JA, Jimenez A, et al. Cadherin-13, a mediator of calcium-dependent cell-cell adhesion, is silenced by methylation in chronic myeloid leukemia and correlates with pretreatment risk profile and cytogenetic response to interferon alfa. J Clin Oncol 2003;21:1472–9.
36. Sato M, Mori Y, Sakurada A, Fujimura S, Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum Genet 1998;103:96–101.
37. Yan PS, Rodriguez FJ, Laux DE, et al. Hypermethylation of ribosomal DNA in human breast carcinoma. Br J Cancer 2000;82:514–7.
38. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science (Wash DC) 2003;300:455.
39. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. Science (Wash DC) 2003;300:489–92.
40. Hernandez R, Frady A, Zhang XY, Varela M, Ehrlich M. Preferential induction of chromosome 1 multibranched figures and whole-arm deletions in a human pro-B cell line treated with 5-azacytidine or 5-azadeoxycytidine. Cytogenet Cell Genet 1997; 76:196–201.
41. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature (Lond) 1998;395:89–93.
42. Tuck-Muller CM, Narayan A, Tsien F, et al. DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. Cytogenet Cell Genet 2000;89:121–8.
43. Tsien F, Fiala ES, Youn B, et al. Prolonged culture of normal chorionic villus cells yields ICF syndrome-like chromatin decondensation and rearrangements. Cytogenet Genome Res 2002;98:13–21.
44. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature (Lond) 1998;396:643–9.
45. Mitelman F, Mertens F, Johansson B. A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. Nat Genet 1997;15:417–74.
46. Jeanpierre M, Turleau C, Aurias A, et al. An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 1993;2:731–5.
47. Kokalj-Vokac N, Almeida A, Viegas-Pequignot E, et al. Specific induction of uncoiling and recombination by azacytidine in classical satellite-containing constitutive heterochromatin. Cytogenet Cell Genet 1993;63:11–5.
48. Ji W, Hernandez R, Zhang XY, et al. DNA demethylation and pericentromeric rearrangements of chromosome 1. Mutat Res 1997;379:33–41.
49. Wong N, Lam WC, Lai PB, et al. Hypomethylation of chromosome 1 heterochromatin DNA correlates with q-arm copy gain in human hepatocellular carcinoma. Am J Pathol 2001;159:465–71.
50. Schulz WA, Elo JP, Florl AR, et al. Genomewide DNA hypomethylation is associated with alterations on chromosome 8 in prostate carcinoma. Genes Chromosomes Cancer 2002;35:58–65.
51. Tsuda H, Takarabe T, Kanai Y, Fukutomi T, Hirohashi S. Correlation of DNA hypomethylation at pericentromeric heterochromatin regions of chromosomes 16 and 1 with histological features and chromosomal abnormalities of human breast carcinomas. Am J Pathol 2002;161:859–66.
52. Gupta A, Godwin AK, Vanderveer L, Lu A, Liu J. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. Cancer Res 2003;63:664–73.
53. Scelfo RA, Schwienbacher C, Veronese A, et al. Loss of methylation at chromosome 11p15.5 is common in human adult tumors. Oncogene 2002;21:2564–72.
54. Cho M, Uemura H, Kim SC, et al. Hypomethylation of the MN/CA9 promoter and up-regulated MN/CA9 expression in human renal cell carcinoma. Br J Cancer 2001;85:563–7.
55. Sato N, Maitra A, Fukushima N, et al. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res 2003;63:4158–66.
56. Gasser SM. Positions of potential: nuclear organization and gene expression. Cell 2001;104:639–42.
57. Sabbattini P, Lundgren M, Georgiou A, et al. Binding of Ikaros to the lambda5 promoter silences transcription through a mechanism that does not require heterochromatin formation. EMBO J 2001;20:2812–22.
58. Aparicio A, Weber JS. Review of the clinical experience with 5-azacytidine and 5-aza-2′-deoxycytidine in solid tumors. Curr Opin Investig Drugs 2002;3:627–33.
59. Stoner GD, Shimkin MB, Kniazeff AJ, et al. Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice. Cancer Res 1973;33:3069–85.
60. Carr BI, Reilly JG, Smith SS, Winberg C, Riggs A. The tumorigenicity of 5-azacytidine in the male Fischer rat. Carcinogenesis (Lond) 1984;5:1583–90.
61. Kerbel RS, Frost P, Liteplo R, Carlow DA, Elliott BE. Possible epigenetic mechanisms of tumor progression: induction of high-frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. J Cell Physiol 1984;3(Suppl):87–97.
62. Sessa C, ten Bokkel HW, Stoter G, Renard J, Cavalli F. Phase II study of 5-aza-2′-deoxycytidine in advanced ovarian carcinoma. The EORTC Early Clinical Trials Group. Eur J Cancer 1990;26:137–8.