Abstract

**Purpose:** To identify novel endometrial cancer-specific methylation markers and to determine their association with clinicopathologic variables and survival outcomes.

**Experimental Design:** Differential methylation hybridization analysis (DMH) was done for 20 endometrioid endometrial cancers using normal endometrial DNA as a reference control. Combined bisulfite restriction analysis (COBRA) was used to verify methylation of sequences identified by DMH. Bisulfite sequencing was undertaken to further define CpG island methylation and to confirm the reliability of the COBRA. The methylation status of newly identified markers and the MLH1 promoter was evaluated by COBRA in a large series of endometrioid (n = 361) and non-endometrioid uterine cancers (n = 23).

**Results:** DMH and COBRA identified two CpG islands methylated in tumors but not in normal DNAs: SESN3 (PY284) and TITF1 (SC77F6/154). Bisulfite sequencing showed dense methylation of the CpG islands and confirmed the COBRA assays. SESN3 and TITF1 were methylated in 20% and 70% of endometrioid tumors, respectively. MLH1 methylation was seen in 28% of the tumors. TITF1 and SESN3 methylation was highly associated with MLH1 methylation (P < 0.0001). SESN3 and TITF1 were methylated in endometrioid and non-endometrioid tumors, whereas MLH1 methylation was restricted to endometrioid tumors. Methylation at these markers was not associated with survival outcomes.

**Conclusions:** The 5’ CpG islands for SESN3 and TITF1 are novel cancer-specific methylation markers. Methylation at these loci is strongly associated with aberrant MLH1 methylation in endometrial cancers. SESN3, TITF1 and MLH1 methylation did not predict overall survival or disease-free survival in this large cohort of patients with endometrioid endometrial cancer.

Endometrial cancer is the most commonly diagnosed malignancy of the female reproductive tract in the United States. Approximately 39,080 new cases of cancer of the uterine corpus will be diagnosed, and 7,400 women will die of this disease in 2007 (1). More than 25% of endometrial cancers show instability of microsatellite DNA repeats (MSI), indicating defective DNA mismatch repair. The most frequent cause of MSI is epigenetic silencing of the MLH1 locus, associated with promoter methylation (2–5).

Methylation of the 5’-carbon in cytosine residues is the most common base modification in the human genome (6). Methylcytosine (5meC) occurs primarily in the context of “CpG” dinucleotides. Approximately 20% of CpG sites are unmethylated and clustered in relatively short (<5 kb) sequences referred to as “CpG islands.” These islands are frequently evolutionary conserved and are associated with more than 50% of human genes. For some CpG islands, methylation increases with age and thereby, in part, reflects the number of cell divisions. Importantly, CpG island methylation has been implicated in silencing or down-regulating genes in cancer (7–9). It has been suggested that cancers can be classified according to their degree of methylation. Extensive CpG island methylation in tumors may reflect a “CpG Island Methylator Phenotype” (CIMP) that in some cases is associated with distinctive pathogenesis, clinical characteristics, and prognosis (10–13).

The importance of MLH1 promoter methylation in endometrioid endometrial cancer and our previous work suggesting the existence of a methylator phenotype in these tumors (14) led us...
to hypothesize that concurrent methylation at other loci might characterize a subset of endometrial cancers. We considered the possibility that tumor-specific methylation at multiple sites might point to a molecular subtype of cancers with distinctive biological and/or clinical characteristics. Therefore, we set out to identify endometrial cancer-specific methylation markers and to determine whether methylation at these sites is correlated with clinical and pathologic variables in endometrioid endometrial carcinomas.

Materials and Methods

All the human tissue specimens used in this study were obtained as part of our ongoing work aimed at characterizing molecular alterations in endometrial cancer. All participants consented to molecular analyses and follow-up. These protocols have been approved by the Washington University Medical Center’s Human Studies Committee.

Differential methylation hybridization analysis. Procedures used for tissue DNA extraction have been described previously (15). Differential methylation hybridization (DMH) analysis was done using high-density slide arrays containing 7,776 CpG island clones (MseI CpG inserts) using 20 endometrial cancer (endometrioid histology, >70% neoplastic cellularity) and three pooled normal endometrium DNAs as a reference control. Tumor and control amplicons were labeled with Cy5 and Cy3, respectively, and cohybridized onto microarray slides using our established protocols (16). Normalized Cy5/Cy3 hybridization intensities were calculated, and candidate loci with ratios >1.5 were scored as hypermethylated in tumors compared with normal endometrial tissues according to previously published methods (17).

Combined bisulfite restriction analysis. DNA bisulfite conversion was done using commercially available kits (CpGenome DNA Modification Kit, Intergen Company; EZ DNA Methylation Gold Kit, Zymo Research) following manufacturers’ recommended protocols.

The combined bisulfite restriction analysis (COBRA) method was used to evaluate methylation (18). All loci with Cy5/Cy3 DMH hybridization ratios of >1.5 suggesting hypermethylation in tumors were considered for COBRA analyses. COBRA assays were not attempted for multicopy sequences and MseI clones lacking either a BstUI or HpaII site required for DMH detection. Briefly, target sequences were amplified using two rounds of PCR. Restriction digestions were then done on PCR products using enzymes that recognize sequences potentially altered by methylation. The sequences for the MseI CpG island clones (19) were extracted from the National Center for Biotechnology Information library or, when not available, determined by sequencing the original clone used in constructing the CpG arrays. PCR primers and conditions, as well as restriction enzymes for the MLH1 promoter, have been published previously (14). Primer sequences, PCR conditions, and restriction enzymes for newly developed assays are presented in Supplementary Table S1.

Unrestricted and restricted PCR products were resolved on 10% non-denaturing polyacrylamide gels, stained with ethidium bromide, and photographed with a UV camera (ImageStore 7500 Version 7.12, White/UV Transilluminator; UVP, Inc.). A sample was classified as methylated if methylation-specific restriction fragments were shown. Two enzyme digestions per locus were done for the majority of assays, including BstUI (CCCG) whenever possible.

Cloning and sequencing of bisulfite-converted DNAs. PCR reactions were done essentially as for the restriction analyses (COBRA). Amplification products were purified using the QiAquick PCR purification kit (Qiagen) and cloned into the pCR-2.1TOPO TA cloning vector (Invitrogen). Following transformation, recombinants were analyzed by PCR (vector-specific primers) to identify clones with the expected size inserts. Bacterial clones were grown overnight, and plasmid DNA was prepared using the QiAprep Spin Miniprep kit (Qiagen). Six clones for each cloning experiment were sequenced using ABI Prism BigDye Terminator chemistry v1.1 (Applied Biosystems).

Methylation typing by COBRA in a large cohort of endometrioid carcinomas. Since 1991, we have prospectively acquired tumor samples from hysterecomy specimens from patients with suspected uterine cancer treated by our Division of Gynecologic Oncology. None of these patients received preoperative radiation or chemotherapy. Surgical staging and tumor grade were assigned based on the International Federation of Gynecology and Obstetrics (FIGO) criteria. Clinical and pathologic data for these cases were prospectively collected and stored in a computerized database. Specific initial treatments were individualized. At the completion of their initial treatment, these patients were routinely followed at 3-month intervals. Standard surveillance included physical examination and Pap smears for at least 2 years after initial treatment. Assessment with additional imaging studies and directed biopsies were done as clinically indicated at the discretion of the treating physician. Recurrent disease was histologically confirmed as indicated.

A total of 930 specimens were included in our tumor bank between November 1991 and January 2005. Histologic slides were uniformly evaluated by experienced gynecologic pathologists at our institution. A total of 106 cases were excluded due to uncertain origin and/or presence of synchronous malignancies. Another 202 cases were excluded due to non-endometrioid histology (25 clear cell carcinomas, 38 papillary serous, 64 mixed histotypes, 7 undifferentiated carcinomas, 2 neuroendocrine tumors, 44 carcinomas; and, 22 sarcomas). Tumor DNA was prepared from flash-frozen tissue (>70% neoplastic cellularity) as previously described (2). Of the remaining 622 endometrioid endometrial cancer cases, bisulfite-converted DNA and follow-up data were available for 361. COBRA was used to evaluate methylation in this cohort and in 23 non-endometrioid uterine tumors.

Statistical analysis. The relationship between methylation and relevant clinicopathologic covariates was done using χ2 and Student’s t test as appropriate. Overall survival (OS) was defined as the time from initial surgery to the date of death due to any causes. Survivors were censored at the date of last contact. Disease-free survival (DFS) was defined as the time from surgery to recurrence or progression. Survival was evaluated with the Kaplan-Meier method. Univariate and multivariate Cox proportional hazard models were fitted to assess the predictive effects of the covariates on OS and DFS. Survival distributions were compared using the log-rank test. All analyses were two-sided, and significance was set at a P value of 0.05. Statistical analyses were done using SAS (SAS Institute).

Results

TTF1 and SSEN3: two novel methylation markers in endometrial carcinoma. Initial microarray data analysis revealed 93 loci with increased levels of methylation in tumors compared with normal endometrial tissues (normalized Cy5/Cy3 > 1.5). A total of 19 of the 20 tumors profiled were presumed sporadic cases. One tumor arose in an MSH2 mutation carrier. COBRA assays were successfully developed and completed for 35 of the MseI CpG clone sequences that seemed to be hypermethylated based on DMH (Supplementary Table S1). COBRA assays were not devised for repetitive elements (multicopy sequences), clone sequences that did not contain either a BstUI (CCCG) or HpaII (CCCG) site required for the initial DMH analysis, and/or for those loci for which primers could not be designed due to density of CpGs. For a subset of assays, attempted PCR failed to give rise to the expected products (complete failures or nonspecific amplification). The 20 tumors used for initial DMH analysis were assessed with COBRA using the appropriate restriction endonuclease digestions to verify the DMH results. Of the 35
CpG-rich regions evaluated, 20 (57%) showed methylation in multiple tumors (range, 7 of 20 to 20 of 20 evaluated). The MLH1 promoter region that is methylated in tumors is within a large MseI restriction fragment (1,117 bp). This sequence would not have been cloned at high efficiency when the CpG island library was constructed. The MLH1 CpG island did not appear on the array, and consequently, we could not measure MLH1 methylation by DMH. We assessed the methylation status for the 20 methylated CpG loci in the matched normal blood DNAs from the 20 endometrial cancer cases. The majority (17 loci) showed methylation in the normal blood DNA. In some instances, all blood DNAs were heavily methylated, whereas others showed limited methylation in all or a subset of the blood DNAs (data not shown). Three sequences (SC11B9, PY2B4, and SC77F6/154) were methylated in tumors but not in normal DNAs (Table 1).

We next validated the cancer-specific methylation pattern of these three sequences by analyzing 20 additional endometrial cancers and matched bloods and 12 normal tissue DNAs: cervix (2), myometrium (2), endometrium (2), fallopian tube (2), spleen (2), and sperm (2; data not shown). All three CpG-rich sequences showed methylation only in tumor tissues. Two of the three MseI clones exhibiting cancer-specific methylation (PY2B4 and SC77F6/154) were associated with recognized CpG islands at the 5’ ends of genes. Clone PY2B4 corresponds to a CpG island located in the promoter region of the SESN3 gene. The COBRA assay used to assess methylation extends from -1,186 to -1,402 bp upstream of exon 1. Clone SC77F6/154 is from the CpG island at the 5’ end of TITF1. This CpG-rich region represented by the COBRA assay is located -1,602 to -1,378 bp 5’ of TITF1’s exon 1. The SC11B9 sequence is poor in CpG dinucleotide pairs (only 14 in the 535-bp MseI clone) and is not associated with a CpG island. Figure 1A illustrates the relationship between the PY2B4 and SC77F6/154 MseI clones and the COBRA assays that revealed the cancer-specific methylation. Figure 1B and C shows representative examples of restriction digests for the PY2B4 and SC77F6/154 loci in tumors and matched normal DNAs. Methylation at the PY2B4 locus was evaluated by BstUI and Hinfl digestions (Supplementary Table S1 and Fig. 1B). Additional analysis with MaeII and TaqI (TCGA), which produced multiple fragments, revealed high concordancy with the BstUI and Hinfl digestions.

**Table 1. Patterns of methylation based on DMH and COBRA verification**

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**NOTE:** +, hypermethylation in the tumors compared with the pooled normal endometrium control by DMH and/or by COBRA for tumors.* methylated seen in endometrial cancers and not in normal tissues.
(data not shown). As shown for the SC77F6/154 locus (Fig. 1C), the patterns of methylation in tumors revealed by COBRA were variable. Some tumor DNAs were extensively methylated (218T, Fig. 1C), whereas others showed much less methylation (1089T, Fig. 1C). Digestion of the SC77F6/154 sequences with \( HinfI \) (GANTC) and \( MaeII \) (ACGT) in a subset of tumors showed methylation patterns concordant with \( BstUI \) and \( Sau3AII \) digestions (data not shown). The matched normal DNAs, on the other hand, did not reveal methylation. One blood DNA (1185N, Fig. 1C) showed trace methylation at the \( Sau3AII \) site (GATC*), but no methylation at either of the \( BstUI \) sites.

Methylation was quantitatively assessed by cloning and sequencing bisulfite-converted DNAs from representative unmethylated normal tissues, unmethylated tumors, and methylated tumors. Results for bisulfite sequencing of \( PY2B4 \) and \( SC77F6/154 \) (six clones) for these representative specimens are presented in Fig. 2. Bisulfite sequencing confirmed the high reliability of the COBRA assays to reflect the overall methylation status of the regions of interest. \( PY2B4 \) methylation proved to be highly tumor specific given how infrequently methylation was seen in normal tissues (Fig. 2A). On the other hand, \( SC77F6/154 \) (Fig. 2B) showed a more widespread methylation pattern. The percentage of CpG pairs methylated at \( SC77F6/154 \) in COBRA-positive tumors ranged from 25% to 72%. All four tumors showed methyl-cytosines at both \( BstUI \) sites and the \( Sau3AII \) site evaluated (Fig. 2B). The two matched normal bloods showed very little methylation (6.9% and 9.8%). Two endometrial hyperplasia specimens (Hyper1060 and Hyper1066) showed patchy methylation involving 46% and 31% of CpG pairs, whereas normal endometrium had much less methylation (3% and 28%). Nonetheless, methylation at the \( SC77F6/154 \) locus remained the highest in COBRA-positive tumors. Based on the high consistency for COBRA and bisulfite analysis of methylation at the \( PY2B4 \) and \( SC77F6/154 \) loci, we went on to methyl-type a large collection of primary endometrial cancer DNAs.

Methylation status of TITF1 and SESN3 in a large cohort of endometrioid carcinomas highly correlates with MLH1 aberrant methylation. We determined the methylation status of the SESN3 (\( PY2B4 \)) and TITF1 (\( SC77F6/154 \)) promoter regions by COBRA in DNA extracted from a series of endometrioid endometrial carcinomas (\( N = 361 \)). The median follow-up time for this cohort was 58.6 months (range, 0.7-171.6). A total of 128 tumors (35%) showed microsatellite instability (MSI). The demographic and disease characteristics for this cohort are presented in Table 2.
MLH1 methylation analysis was done previously for this group of tumors. MLH1 was methylated in 101 cases (28%). The SESN3 and TITF1 promoters were methylated in 71/361 (19.7%) and 250/359 cases (69.6%), respectively (Fig. 3A). TITF1 methylation status could not be determined in two cases. Overall, 86 cases (24%) did not show methylation for any of the three markers analyzed. A total of 157 cases (43.7%) were methylated at one marker, 83 cases (23.1%) were methylated at two markers, and 33 cases (9.2%) were methylated at all three markers (Fig. 3B). Methylation status at SESN3 and TITF1 seemed to be correlated ($P = 0.03$). Furthermore, methylation at these two novel markers was strongly associated with MLH1 promoter methylation (both $P < 0.0001$).

Association between methylation status and clinicopathologic variables. We evaluated the association between methylation of the studied markers and age, race, body mass index (BMI), tumor stage, FIGO grade, histologic type, and patient outcome (OS and DFS).

In our cohort of endometrioid carcinomas ($N = 361$), SESN3 methylation was not associated with patient age ($P = 0.712$). In contrast, methylation of the MLH1 and TITF1 promoters was associated with older age at cancer diagnosis ($P = 0.0004$ and $P = 0.0145$, respectively).

In general, methylation at MLH1, TITF1, and SESN3 was not associated with race ($P = 0.842$, $P = 0.790$, and $P = 0.444$, respectively).

As shown previously by our group (20), MLH1 methylation was associated with lower BMI ($P = 0.0035$). On the other hand, methylation at TITF1 and SESN3 was not associated with BMI ($P = 0.121$ and $P = 0.464$, respectively).

SESN3 methylation status was marginally associated with surgical stage ($P = 0.034$), whereas MLH1 and TITF1 methylation were not associated with stage ($P = 0.401$ and $P = 0.864$).

We noted that MLH1 methylation was associated with higher FIGO grade ($P = 0.003$), whereas TITF1 methylation seemed associated with lower FIGO grade ($P = 0.006$). SESN3 methylation was not associated with FIGO grade ($P = 0.493$).

Methylation at MLH1, SESN3, and TITF1 was not associated with the use of adjuvant treatment ($P = 0.729$, $P = 0.738$, and $P = 0.431$).

MLH1 promoter methylation is a distinctive feature of endometrioid carcinomas (4, 5). We evaluated the methylation status of MLH1, SESN3, and TITF1 in a small cohort of non-endometrioid uterine cancers ($n = 23$; 7 papillary serous carcinomas, 1 mixed papillary serous and endometrioid, 9 clear cell carcinomas, and 6 sarcomas). The mean age (± SD) at the
We next examined associations between clinicopathologic variables and methylation of MLH1, SESN3, and TITF1 in our cohort (N = 361) and OS and DFS. As expected, previously recognized prognostic variables such as age [hazard ratio (HR), 1.052; 95% confidence interval (95% CI), 1.028–1.076], FIGO grade (22%) cases for SESN3 and in 15 cases (65%) for TITF1.

Discussion

Epigenetic regulation of gene expression associated with DNA methylation occurs during normal and abnormal time of diagnosis in this group was 68.6 ± 9.5 years. BMI was available for 9 cases and ranged from 22.90 to 58.86 (mean 36.14). Four cases were stage I (17%), six were stage II (26%), nine were stage III (39%), three were stage IV (13%), and one case was not surgically staged (4%). As expected, the MLH1 promoter region was unmethylated in all 23 cases. In this series of non-endometrioid tumors, methylation was shown in 5 (22%) cases for SESN3 and in 15 cases (65%) for TITF1.

Our previous work has suggested the existence of a CIMP in endometrioid endometrial adenocarcinomas (14). We have now identified two novel markers highly methylated in these tumors: PY2B4 and SC77F6/154. These markers were identified through an evaluation of more than 7,000 candidate CpG island clones (16). Both COBRA and bisulfite sequencing confirmed that methylation in normal tissues was infrequent for PY2B4 and SC77F6/154. These markers are within CpG islands located in the promoter regions of SESN3 (PY2B4) and TITF1 (SC77F6/154). SESN3 is a member of the sestrin family. The sestrins are PA26 related and p53 responsive. They function in DNA damage and growth arrest responses (32, 33). The thyroid transcription factor 1 (TITF1, TITF1/NKX2.1), also known as thyroid/enhancer binding protein (T/EBP), belongs to the NK-2 family and was originally recognized as a binding factor to the thyroglobulin promoter (34). TITF1 is also expressed during the development in the forebrain and lung (35, 36). Expression in these sites is retained in adult life. This allows distinguishing, for example, primary lung adenocarcinomas from metastatic lesions (37). We have shown by reverse transcription-PCR that TITF1 is expressed in normal endometrium and a small subset (~10%) of endometrial cancers (data not shown). There was no obvious association between methylation and TITF1 expression in tumors.

Methylation of the MLH1 promoter is a well-recognized phenomenon in endometrial cancer. In fact, it represents the most common cause of microsatellite instability encountered in sporadic cases and the best characterized epigenetic alteration in this malignancy (3–5). In our study of 361 endometrioid carcinomas, methylation at SESN3 and TITF1 was highly associated with MLH1 methylation (both P < 0.0001). In this series, 44% of tumors were methylated in at least one marker, and in 32% of cases, methylation was confirmed on two or more of these markers. The association between SESN3, TITF1,
and MLH1 methylation suggests a pattern of clustered methylation in endometrioid tumors. Similar patterns of clustered methylation have been recently reported in MLH1-methylated colorectal carcinomas favoring the existence of CIMP-positive tumors (38).

In other malignancies, the methylator phenotype has proven to be biologically and/or clinically relevant in identifying special tumor subsets with particular characteristics (13, 21–28). We found that TITF1 methylation was associated with older age ($P = 0.0145$) and with lower FIGO grade ($P = 0.006$). The reason behind these observations remains elusive. It is known that age-related methylation in general reflects the number of cell divisions (9). Mitotic rates in cancer are not only highly variable but also difficult to estimate in studies such as ours. Therefore, it is extremely difficult to assess what influence the number of cell divisions may have in defining methylation patterns in individual tumors.

As previously described (2, 39), MLH1 methylation in the present study seemed restricted to endometrioid tumors. Unlike MLH1, SESN3 and TITF1 methylation was also seen in endometrial cancers of non-endometrioid histology (22% and 65% of cases, respectively).

In this study, we could not show an association between methylation at the novel SESN3 and TITF1 markers or MLH1 and survival outcomes in patients with endometrioid endometrial cancer.

In general, the molecular events associated with tumor methylation hold promise for cancer risk assessment, diagnosis, and prognosis. Moreover, epigenetic abnormalities (specifically gains in methylation) are potentially reversible phenomena that represent an attractive target for therapeutic strategies (7). Several methylation markers have been identified in endometrial cancer: MLH1, HOXA11, THBS2, CDH13, HSPA2, RASSF1A, SOCS2, PER1, RARB2, GSTP1, SFN (14-3-3 sigma),

<table>
<thead>
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<th>Table 3. Methylation and OS and DFS in patients with endometrioid endometrial cancer</th>
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HOXA10 and now SESN3 and TTFI (4, 5, 14, 40–46). In general, most markers seem cancer specific and highly informative. If validated, it might be possible to develop strategies that combine the use of these markers for diagnostic purposes.

To this date, biological significance and clinical impact are only well understood for methylation of the MLH1 promoter region in endometrial cancer. Additional studies to further address the significance of other methylation markers in this disease are warranted. More importantly, potential clinical implications of the methylator phenotype for diagnosis, prognosis, and treatment of endometrial cancers should be explored.

Acknowledgments

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References

Clinical Cancer Research

Differential Methylation Hybridization Array of Endometrial Cancers Reveals Two Novel Cancer-Specific Methylation Markers

Israel Zighelboim, Paul J. Goodfellow, Amy P. Schmidt, et al.


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