Frequent HOXA11 and THBS2 Promoter Methylation, and a Methylator Phenotype in Endometrial Adenocarcinoma

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ABSTRACT

Purpose: This study was designed to determine whether there is a methylator phenotype in stage I and II endometrial endometrial adenocarcinoma, and if so, whether methylation correlates with recurrence.

Experimental Design: Bisulfite-converted DNAs from 24 stage I and II primary cancers (12 recurrent and 12 nonrecurrent), and 5 endometrial cancer cell lines were analyzed for methylation in the promoter regions of seven genes. A methylation index (MeI) was calculated for each tumor. Frequent HOXA11 and THBS2 methylation prompted analysis of case-matched bloods and 25 additional nonrecurrent primary cancers. Statistical analysis included Fisher’s exact and Student t tests.

Results: Rates of methylation in the initial tumor series were as follows: HOXA11, 70.8%; THBS2, 62.5%; MLH1, 33.3%; CTNNB1, 16.7%; VDR, 4.2%; CDKN2A, 4.2%; and THBS1, 0%. There was no difference in the MeI of recurrent and nonrecurrent cases. However, cell lines had higher mean MeI. High rates of HOXA11 and THBS2 methylation were confirmed in the additional nonrecurrent tumors. None of the 24 case-matched bloods had HOXA11 methylation, whereas three blood DNAs showed THBS2 methylation. There was a statistically significant difference in the rate of HOXA11 methylation in recurrent and nonrecurrent tumors (P = 0.0167).

Conclusions: Endometrial adenocarcinomas have a methylator phenotype. No correlation between MeI and clinicopathologic variables in early stage tumors was observed. High rates of methylation were found in the HOXA11 and THBS2 promoter regions. HOXA11 promoter methylation was significantly more frequent in recurrent than nonrecurrent cases. HOXA11 methylation in early stage endometrial cancer is associated with poor outcome.

INTRODUCTION

Uterine corpus cancer is the most common gynecologic malignancy in the United States and the fourth most common cancer in women. It is estimated that there will be 39,300 newly diagnosed cases of endometrial cancer with 6,600 deaths in 2002 (1). Endometrioid endometrial adenocarcinoma is by far the most common form of endometrial cancer, accounting for 87% of all uterine malignancies. Eighty-four percent of endometrioid endometrial adenocarcinomas present as stage I or II disease (2). Although risk of recurrence is low for women with early stage endometrioid endometrial adenocarcinoma, there is significant morbidity and mortality associated with this form of disease because of its overall high incidence. Treatments for endometrial cancer recurrence are limited in their success. Five-year survival after nonvaginal recurrence is 13%, and median survival after recurrence is 10 months (3, 4).

Prolonged or unopposed estrogenic stimulation of the endometrium and genetic predisposition (hereditary nonpolyposis colorectal cancer) are potent risk factors for developing endometrial adenocarcinoma. Genetic abnormalities in DNA mismatch repair genes, tumor suppressors, and oncogenes contribute to endometrial tumorigenesis. Mutations in PTEN, TP53, V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homologue, and CTNNB1,3 loss of DNA mismatch repair, and hypermethylation of promoters of genes such as estrogen receptor, androgen receptor, and MLH1 have all been implicated in endometrial tumorigenesis (5–15).

Clinicopathologic and molecular features of the primary tumor that correlate with poor prognosis include lymphatic vascular space involvement, nonendometrioid histology, higher stage, higher grade, myometrial invasion, positive peritoneal cytology, lymph node metastasis, adnexal metastasis, and aneuploidy (16–20). MSI, and TP53, THBS2, and MLH1 expression have also been associated with outcome (21–26).

Epigenetic changes, specifically hypo- and hypermethylation, are known to be important in tumorigenesis (27–30). Methylation of CpG pairs within CG-rich promoter regions negatively affects expression (31). Not surprisingly, hypermethylation of the promoter region of genes involved in cell cycle control, cell adhesion, apoptosis, angiogenesis, mismatch

Received 9/4/02; revised 1/13/03; accepted 1/14/03.

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1 Supported by CA71754 (to P. J. G.) and a Glaxo SmithKline Oncology Gynecologic Oncology Fellowship at Washington University School of Medicine.

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3 The abbreviations used are: CTNNB1, β-catenin; MSI, microsatellite instability; THBS, thrombospondin; CIMP, CpG island methylator phenotype; HOXA11 homobox A11; VDR, vitamin D receptor (25-hydroxvitamin D3–1α-hydroxylase); MLH1, mutL DNA mismatch repair gene; CDKN2A, cyclin-dependent kinase inhibitor 2A; COBRA, combined bisulfite restriction analysis; MeI, methylation index.
repair, or other key regulatory functions have been implicated in gene silencing and tumorigenesis (27, 29, 30). The methylation pattern of multiple genes has also been correlated with prognosis in specific malignancies (32, 33).

A CIMP was initially described in colorectal cancers (34). Analysis of a panel of CpG-rich sequences revealed that a fraction of primary cancers had frequent promoter hypermethylation. The so-called CIMP+ tumor classification (defined as ≥5 of 7 loci with methylated promoters) was based on analysis of loci known to be methylated in MSI-positive colorectal cancers. It is proposed that the CIMP+ tumors have a global promoter hypermethylation pattern that contributes to tumorigenesis (34). Increases in methylation, measured and expressed in terms of a methylator phenotype or MeI, have been reported subsequently for other cancer types. In a number of instances the methylator phenotype has been correlated with clinicopathologic features and/or prognosis (32–41). The purpose of the present study was to determine whether there is a methylator phenotype in endometrioid endometrial cancer, and, if so, whether methylation patterns are associated with recurrence in low- and intermediate-risk (stage I and II) disease. The genes selected for promoter methylation analysis in this study were in part chosen based on their potential involvement with endometrial tumorigenesis. MLH1 is a mismatch repair gene that has been shown previously to have a high degree of promoter methylation in endometrial cancers (12, 42). Likewise, CDKN2A (p16INK4a) is a tumor suppressor gene that has been shown to have promoter methylation in endometrial cancers (43). These two genes could serve as positive controls for promoter methylation. The thrombospondins are known to be angiogenesis inhibitors and/or promoters, and, therefore, are important factors in tumor proliferation (44, 45). TSP1 methylation has been demonstrated in other tumor types, and TSP2 expression is correlated with poor outcome in endometrial cancers (21). HOXAI1 methylation has not been evaluated previously in human endometrial cancers. This gene, and other homeobox genes, are expressed in endometrial epithelium, and are known to play a role in uterine embryogenesis (46, 47). It is postulated that altered HOXAI1 expression in the adult female may subsequently lead to aberrant endometrial proliferation and possible tumorigenesis. VDR is involved with antiproliferation and prodifferentiation (48). Decreased expression of its product has been seen in endometrial cancers (49); however, methylation as a proposed mechanism has not been evaluated. CTNNB1 participates in the tissue adhersens complex. Mutation in this gene is seen in 15% of endometrial cancers and is associated with higher tumor grade; however, this is not known to be a tumor suppressor (10, 11). One would not expect methylation-related expression changes in this gene, and as such, CTNNB1 could serve as a negative control for methylation. A biomarker to identify patients who are at increased risk for recurrence and for whom aggressive frontline therapy might be beneficial could ultimately lead to improved disease-specific survival. Methylation in the 5′ promoter regions of seven genes was investigated in a panel of primary endometrial adenocarcinomas and endometrial cancer cell lines to determine whether there is a methylator phenotype, and whether patterns of methylation are associated with outcome.

MATERIALS AND METHODS

Cell Line, Primary Tumor, and Normal DNAs. Promoter methylation was assessed in five endometrial cancer cell lines: KLE, AN3CA, RL95-2, and HEC-1-A (American Type Culture Collection, Manassas, VA), and Ishikawa (Stuart Adler, Washington University, St. Louis, MO), and a panel of 24 early stage primary endometrioid endometrial adenocarcinomas. Tumor DNAs were prepared from primary hysterectomy specimens as described previously (50). Twelve of the tumors were from patients who subsequently recurred. These 12 cases were matched for stage and histological grade with 12 endometrioid adenocarcinomas from women who did not recur (identical stage and grade for 11 of 12, and grade 2 was matched with grade 3 for 1 stage IIB primary tumor). Normal peripheral blood leukocyte DNA from these 24 patients was also evaluated for methylation of HOXAI1 and THBS2. These 24 cases were derived from a larger series described previously by our group (50).

Twenty-seven additional early stage (IA-IIB), nonrecurrent endometrioid endometrial adenocarcinomas, 14 colorectal, 10 breast, and 10 ovarian carcinoma DNAs were evaluated for HOXAI1 and THBS2 methylation. All of the patient materials in this retrospective nested case study were obtained with appropriate Human Studies Committee/Institutional Review Board approval (Washington University School of Medicine Institutional Review Board approvals 93-0828 and 96-0257 for endometrial and colorectal cancers). Breast and ovarian tumor specimens were obtained through the Cooperative Human Tissue Network. The histological subtypes of the breast and ovarian cancers evaluated were not specified.

Bisulfite Conversion. DNAs were subjected to bisulfite conversion using the CpGenome DNA Modification kit (Intergen Company, Purchase, NY) as described previously (51). After bisulfite modification, DNA samples were stored at −20°C.

Methylation Analysis. The CpG-rich 5′ regions of seven genes were assessed for methylation. Five of the genes studied had not been evaluated previously for methylation in endometrial cancers: HOXAI1, THBS1, THBS2, CTNNB1, and VDR. Two genes, MLH1 and CDKN2A, investigated previously in endometrial cancer and other tumors for promoter hypermethylation were also studied. The loci evaluated, including chromosomal locations, National Center for Biotechnology Information accessions, and specific CpG sites analyzed for methylation are shown in Table 1. The COBRA method was used to survey methylation (52).

In brief, sequences of interest were amplified using two rounds of PCR. PCR primers were selected to amplify both methylated and unmethylated sequences (with the exception of the MLH1 inner forward primer, primer sequences do not include methylatable cytosines). The primer sequences and annealing temperature are given in Table 2. The resultant products were digested with restriction enzymes for which the recognition sites (Table 1) reflect the methylation status of the input genomic DNA. PCR products were resolved on 10% nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized...
with UV illumination. The unrestricted and restricted PCR products were run side-by-side. Photomages (ImageStore 7500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA) were then used to quantitate the level of methylation for each digest. The extent of methylation was assessed by densitometric analysis (ImageQuant for Macintosh, Version 1.0; Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). A DNA was considered positive for methylation at a given promoter region if there were methylation-specific restriction fragments with both restriction enzymes, and the average percentage of methylation was \( \geq 10\% \).

**Statistical Analysis.** Statistics were performed using InStat for Macintosh, Version 2.03 (GraphPad Software, Inc., San Diego, CA): Fisher’s exact test was used to determine significance between methylation in recurrent and nonrecurrent primary tumors. Unpaired Student’s \( t \) test was used for comparison between recurrent and nonrecurrent primary tumors for quantitative data for HoxA11 and Thbs2 methylation.

**RESULTS**

**Patterns of Methylation in Primary Endometrial Cancers and Endometrial Cancer Cell Lines.** The frequency of methylation of the seven CpG-rich promoter regions evaluated ranged from 0% for Thbs1 to 70.8% for HoxA11 (Fig. 1). The MeI, calculated by dividing the number of sites methylated by the total number of sites studied in primary tumors. The MeI was higher overall in the cancer cell lines, ranging from 0.14 (KLE) to 0.71 (HEC-1-A). Six of the seven promoters studied had methylation in one or more of the cell lines. Thbs1 did not show methylation in any of the cell lines or primary endometrial cancers. Normal blood specimens from two cancer-free controls were unmethylated for all seven of the promoter regions analyzed (Fig. 1).

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**Table 1** Gene sequences evaluated for methylation in endometrial adenocarcinomas

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene</th>
<th>Chromosomal location</th>
<th>NCBI* accession</th>
<th>Amplimer location*</th>
<th>Cytosines evaluated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXA11</td>
<td>Homeobox A11</td>
<td>7p15-p14</td>
<td>AF071164</td>
<td>3637–3791</td>
<td>TaqII 3679, BstUI 3730/3732, BstUI 3734/3736</td>
</tr>
<tr>
<td>THBS2</td>
<td>Thrombospondin-2</td>
<td>6q27</td>
<td>U79410</td>
<td>4811–4948</td>
<td>TaqII 4872, BstUI 4895</td>
</tr>
<tr>
<td>MLH1</td>
<td>Escheria coli mutL homologue</td>
<td>3p21.3</td>
<td>U26559</td>
<td>601–715</td>
<td>Sau3A I 672, BstUI 684/686, BstUI 858/860, BstUI 859/859, BstUI 905/909, BstUI 922/924, BstUI 960/962, HhaI 858/860, BstUI 960/962</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>( \beta )-catenin</td>
<td>3p22-p21.3</td>
<td>X89448</td>
<td>795–1088</td>
<td>TaqII 357, BstUI 454/456, BstUI 46/48, BstUI 161/163, TaqII 226, 247, BstUI 2257/2259/2261, 2410/2412 TaqII 2291</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor (25-hydroxyvitamin D3-1 ( \alpha ) hydroxylase)</td>
<td>12q13.3</td>
<td>AB005990</td>
<td>302–521</td>
<td>TaqII 357, BstUI 454/456, BstUI 46/48, BstUI 161/163, TaqII 226, 247, BstUI 2257/2259/2261, 2410/2412 TaqII 2291</td>
</tr>
<tr>
<td>CDK2A</td>
<td>Cyclin-dependent kinase inhibitor 2A ( (p16^{INK4a}) )</td>
<td>9p21</td>
<td>U12818</td>
<td>16–273</td>
<td>TaqII 357, BstUI 454/456, BstUI 46/48, BstUI 161/163, TaqII 226, 247, BstUI 2257/2259/2261, 2410/2412 TaqII 2291</td>
</tr>
<tr>
<td>THBS1</td>
<td>Thrombospondin</td>
<td>15q15</td>
<td>J04835</td>
<td>2158–2440</td>
<td>TaqII 357, BstUI 454/456, BstUI 46/48, BstUI 161/163, TaqII 226, 247, BstUI 2257/2259/2261, 2410/2412 TaqII 2291</td>
</tr>
</tbody>
</table>

* Refers to sequences amplified in the second round of PCR, and subjected to restriction analysis.
* NCBI, National Center for Biotechnology Information.
* Refers to locations of nucleotides (cytosines) assessed by restriction digestion.
* HhaI sites evaluated same CpG pairs as BstUI digestion.
Table 2: Primers and conditions for amplification of promoter region for COBRA analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temp (°C)</th>
<th>Outer primers (round 1)</th>
<th>Inner primers (round 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXA11</td>
<td>gTttTTtttTtttgtagTTa</td>
<td>ccaAAAcatccctcacccaccc</td>
<td>48</td>
<td>TTaTTtTaggggaagTaaTaga</td>
<td>aAAaccctAcaattAaAcacaa</td>
</tr>
<tr>
<td>THBS2</td>
<td>tgtataTTTtgaTTtggga</td>
<td>ttAccaacatttatctcaAAc</td>
<td>48</td>
<td>gggtgatgTTtgaggtgtgggag</td>
<td>caaAtccccttAAtAcacactt</td>
</tr>
<tr>
<td>MLH1</td>
<td>tttTtTaaTtTtgtgggttgTtggg</td>
<td>AAaAAccacaaAaAcaAAAccaa</td>
<td>55</td>
<td>TtgTTcg</td>
<td>b</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>ggaggataTTagggTTaTTttTT</td>
<td>AcacctcaAAAAaacaAActcct</td>
<td>48</td>
<td>gtggggtgTTTaggTaTTTTa</td>
<td>AaaActActcctcaAaccttcctc</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>gtagagTaaatgtgggaTag</td>
<td>atAtctAAactAtccatctca</td>
<td>48</td>
<td>ggatgTTTatgaaataaggaa</td>
<td>AAaacccatAtaActttaaAcc</td>
</tr>
<tr>
<td>VDR</td>
<td>gaTttttTtgagaagttTtagtgT</td>
<td>ccaccaAaAAAactAaaAcctca</td>
<td>48</td>
<td>ggagagaggagTTTagaTtggTTT</td>
<td>AAAAcAacttacctAtAtAtacc</td>
</tr>
<tr>
<td>THBS1</td>
<td>gtagagTaaatgtgggaTag</td>
<td>atAtctAAactAtccatctca</td>
<td>48</td>
<td>ggatgTTTatgaaataaggaa</td>
<td>AAaacccatAtaActttaaAcc</td>
</tr>
</tbody>
</table>

* Capitals indicate bases that have been bisulfite converted.

MLH1 and CDKN2A. Two of the loci studied have been evaluated previously for methylation in endometrial adenocarcinoma (MLH1 and CDKN2A). The importance of methylation of the MLH1 promoter and loss of mismatch repair in endometrial cancers is well established (12, 42). In this series, 33% (8 of 24) of the primary tumors demonstrated methylation of the MLH1 promoter. All of the cancers with MLH1 methylation had been shown previously to have MSI (data not shown). CDKN2A promoter methylation in our series was seen in 1 of 24 (4.2%) primary tumors and 2 of 5 (40%) cell lines (Fig. 1). Although only 1 primary tumor was classified as having CDKN2A methylation (methylation of both TaqI and BstUI sites, with a mean level of methylation ≥10%), 6 primary tumors showed some degree of methylation of the CDKN2A promoter. In these cases, there was low-level methylation at both the TaqI and BstUI sites, but at levels substantially <10% average used to classify the locus as methylation-positive. In addition, several tumors showed methylation with a single restriction enzyme digestion.

THBS1 and THBS2. Expression of THBS1 and THBS2 has been evaluated previously in endometrial adenocarcinoma, and THBS2 levels were shown to correlate negatively with outcome (21). The CpG residues we studied in the THBS1 promoter were not methylated in any of the cell lines or primary tumors. The THBS2 promoter region, on the other hand, demonstrated extensive methylation in 15 of 24 (62.5%) primary tumors and 2 of 5 (40%) cell lines. Among the primary tumors with THBS2 methylation, 8 cases were in the recurrence group, and 7 were in the nonrecurrence group (Fig. 1). This difference in THBS2 methylation rates was not statistically significant.

To determine whether THBS2 methylation was a tumorspecific event, the normal cellular DNAs (isolated from peripheral blood leukocytes) from the 24 endometrial cancer patients (12 who recurred and 12 who did not, see Fig. 1) were assessed for methylation. THBS2 promoter methylation was evident in 3 of the matched blood samples (data not shown). These methylated bloods were all in the recurrent group, and all had methylation in the primary tumor as well (cases 0008, 1093, and 0124; see Fig. 1).

CTNNB1. CTNNB1 showed low levels of methylation in 4 of 24 (16.7%) tumors and 2 of 5 (40%) cell lines. All 4 of the cases with CTNNB1 methylation were in the nonrecurrent group.

VDR. VDR methylation was seen at low levels in only 1 of 24 (4.2%) of the primary endometrial tumors. This was in a nonrecurrent case. Two of 5 (40%) cell lines were methylated at the VDR promoter.

HOXA11. The HOXA11 promoter had the highest rate of methylation, with 17 of 24 (70.8%) primary tumors and all of the cell lines showing methylation. Eleven of 12 (91.7%) recurrences and 6 of 12 (50%) nonrecurrences were methylated (P = 0.0686, Fisher’s exact test).

HOXA11 methylation in tumors has not been described previously. To determine whether the methylation we observed is tumor-specific, the normal cellular DNAs (isolated from peripheral blood leukocytes) from the 24 endometrial cancer patients (12 who recurred and 12 who did not, see Fig. 1) were assessed for methylation. None of the normal blood samples...
revealed methylation of both the *TaqI* and *BstUI* sites in the *HOXA11* promoter, and as such, the blood DNAs were classified as unmethylated. Methylation was observed at a single CpG (*TaqI* site) in 4 of 24 of the normal bloods (data not shown). All 4 of the patients with *HOXA11 TaqI* site methylation were in the recurrence group (cases 0008, 0124, 1176, and 1112; see Fig. 1).

**Methylation and Outcome (Recurrence) in Endometrial Cancers.** The primary endometrial adenocarcinomas we studied were all stage I or II. Twelve of the 24 primary tumors evaluated came from women who recurred subsequently.

The average MeI for the recurrent endometrial cancer cases and nonrecurrences was the same (0.28). Most of the methylation seen in these tumors was at the *HOXA11* or *THBS2* loci. Of the 46 examples of methylated promoters seen in the initial 24 primary tumors, 32 (70%) were in *HOXA11* or *THBS2* (see Fig. 1). A tumor with a MeI ≥0.2 has been classified previously in the “high-MeI” group (32). Using this criterion, 75% of cancers in the initial series (18 of 24) would be classified as having high MeI. In this study of endometrial cancer, the most frequent MeI was 0.29 (2 of 7 loci methylated). Six primary tumors had MeI >0.29 (high-MeI), and 6 had MeI <0.29 (low-MeI).

The initial analysis of 24 cases of low-stage (I/II) endometrial adenocarcinoma showed a trend toward increased *HOXA11* methylation in the recurrences versus nonrecurrences. *HOXA11* methylation was assessed in 25 additional low-stage, nonrecurrent primary endometrial tumors. Because of the low rate of recurrence for stage I/II endometrial tumors, there were no additional recurrent cases among our patient population. Of these 25 tumors, 13 (52%) were *HOXA11* methylation-positive (Table 3). In the combined series, 2 tumors (1028 and 1117) showed low levels of methylation at the *TaqI* and *BstUI* sites investigated (6% and 6%, and 6% and 6%, respectively), but did not meet the 10% mean methylation criteria established as the cutoff for classifying the tumor as methylation-positive (Table 3). When the 25 additional primary endometrial cancers were combined with the initial 24 cases, a statistically significant increase in frequency of *HOXA11* methylation in recurrences was evident (11 of 12 recurrences versus 19 of 37 nonrecurrences; *P* = 0.0167, Fisher’s exact test). The level of *HOXA11* methylation was determined for all 49 of the cases. The mean percentage of methylation was calculated for all of the tumors demonstrating any methylation with both enzymes. The mean for recurrences was 40% compared with 37.0% in nonrecurrences (*P* = 0.707, unpaired Student’s *t* test).

*THBS2* methylation was also assessed in the additional nonrecurrent cases. When combined with the initial 24 tumors, *THBS2* methylation was seen in 8 of 12 (66.7%) recurrent primary tumors, compared with 25 of 37 (67.6%) nonrecurrent primary tumors (*P* = 1.0, Fisher’s exact test; Table 3).

To begin to determine whether *HOXA11* and *THBS2* promoter methylation is an endometrial cancer-specific event, we analyzed 14 colorectal, 10 ovarian, and 10 breast primary tumor specimens using the same COBRA assays. Three of 14 (21.4%) colorectal tumors had *HOXA11* methylation, and 1 of 14 (7.1%) had *THBS2* methylation. In ovarian cancers, the methylation rates were 30% for *HOXA11* and 20.0% for *THBS2*; breast cancers had 70% and 55.6% methylation, respectively.

![Fig. 1 Patterns of methylation of HOXA11, THBS2, MLH1, CTNNB1, VDR, CDKN2A, and THBS1 in primary endometrial cancers and endometrial cancer cell lines.](image-url)
DISCUSSION

We evaluated primary endometrial cancers and endometrial cancer cell lines to determine whether endometrioid adenocarcinomas exhibit a methylator phenotype. Methylator phenotypes, measured and described in terms of either the so-called CIMP or MeI have been reported for colorectal, gastric, pancreatic, bladder, prostate, and nasopharyngeal cancers (32–36, 38, 39, 41). The mean MeI for the 24 primary endometrial cancers we studied was 0.28. The MeI for individual tumors ranged from 0.00 (2 cases) to 0.43 (6 cases). The patterns of methylation we observed suggest that there is a methylator phenotype in endometrial cancers. In our panel of low-stage endometrioid adenocarcinomas, MeI did not correlate with clinicopathologic variables. The genes we evaluated had either been associated previously with endometrial cancer prognosis, or represented biologically plausible candidates for playing a role in endometrial tumorigenesis. Five of seven genes studied have not been evaluated previously for methylation in human endometrial cancers. Two of these, HOXA11 and THBS2, were methylated frequently. The patterns of methylation seen in the endometrial cancer cell lines may not reflect the methylation present in the primary tumors from which the cell lines were derived.

MLH1 and CDKN2A methylation has been reported previously for endometrial cancer. The MLH1 mismatch repair gene is methylated frequently in endometrial cancers with MSI (12, 42). Promoter methylation is associated with an absence of MLH1 protein, and is an early event in endometrial tumorigenesis (12, 42, 51). In colorectal cancers, a CIMP has been associated with MLH1 methylation and MSI-positive tumors (53, 54).

CDKN2A (p16 INK4a) is a tumor suppressor gene that demonstrates mutation and promoter methylation in a variety of malignancies. The PCR amplimer and CpGs studied by COBRA incorporated several of the cytosines evaluated previously (55). CDKN2A is an inhibitor of cyclin-dependent kinase 4, which is involved with the modulating phosphorylation of Rb, a potent tumor suppressor gene. Alterations in p16 and the Rb pathway may be early events in endometrioid endometrial tumorigenesis (56, 57). Diminished p16 expression has been correlated with methylation of its promoter (57, 58). Methylation of p16 INK4a was reported for 22% of primary gynecologic malignancies overall and in 16% of low-stage (I-II) endometrial cancers in one investigation (43). Other groups have reported a much lower

Fig. 2 COBRA methylation analysis in primary endometrial cancers. A, HOXA11 partial methylation in all three tumors shown, with nearly full methylation in tumor 0002. B, THBS2 partial methylation in tumors 0008 and 1093, and no methylation in tumor 0002. C, MLH1 methylation in tumor 1087. Tumors 1085 and 1247 are unmethylated. D, CTNNB1 methylation in tumor 1256, and no methylation in 1271 and 1173. E, VDR A number of the tumors in our series had partial, low-level VDR methylation, but did not meet the criteria established for classification as methylation-positive. Tumor 1087 is an example, that shows <10% methylation, and as such was not classified as methylation-positive. Tumor 0008 demonstrates methylation at the TaqI site only, and tumor 1257 has no methylation. F, CDKN2A methylation in tumor 1173 with a combined average methylation ≥10%. Tumors 1271 and 1330 demonstrate restriction with only the TaqI enzyme. G, THBS1 was not methylated in any of the tumors analyzed. T, TaqI; B, BstUI; H, HhaI; R, RsaI; S, Sau3AI; U, unrestricted PCR product. Fragment sizes are indicated in bp, and tumor identifiers are shown above each representative gel.
rate of CDKN2A methylation (0.7–3%; Refs. 59, 60). We found 4.2% of tumors to be methylated as defined by our criteria, which is consistent with previous reports.

Endometrial angiogenesis, modulated by vascular endothelial growth factor, the thrombospondins, and other regulators, is known to play an important role during endometrial proliferation in the normal human endometrial cycle (44, 45). THBS1, regulated by the TP53 and Rb tumor suppressors, can act to promote or suppress angiogenesis and fibrinolysis (21, 61). It is also known to be up-regulated, predominantly in stromal cells,
by progesterone in the human endometrium, and function in this role as an angiogenesis suppressor during the secretory phase of the menstrual cycle (45). Because angiogenesis is crucial for tumor growth, abnormal expression of THBS1 can be a requirement for such a lesion to develop. THBS1 methylation has been evaluated previously in other cancers, such as glioblastoma multiforme, prostate, lung, hematopoietic, and colorectal (61, 62). Presence or absence of expression has not correlated with prognosis in endometrial cancer (21, 63).

THBS2 function is largely unknown, but THBS2 has a similar structure to THBS1. In endometrial carcinoma, THBS2 was found to have expression directly correlated with lymphatic vascular space involvement and cervical involvement, and inversely related to disease-free survival in endometrial cancer (21). Promoter methylation of THBS1 and THBS2 has not been evaluated previously in endometrial cancer. In our series, none of the cancers or cell lines were shown to be methylated in the THBS1 promoter region evaluated. The region evaluated overlaps with the sequences investigated by Li et al. (61) in other tumor types. THBS2 promoter methylation, on the other hand, occurred in a high percentage of primary endometrial cancers (33 of 49; 67.3%). To the best of our knowledge, THBS2 methylation in tumors has not been reported previously.

CTNNB1 is a component of the tissue adherens complex involved with the cadherins and other catenins, which resides in the cell membrane, cytoplasm, and nucleus normally at low levels (64). CTNNB1 mutations are present in ~15% of endometrioid endometrial cancers (10, 11). CTNNB1 maps to 3p22-p21.3, a region that has been reported to show frequent allelic deletion in endometrial cancers (65, 66). In endometrioid adenocarcinomas, allelic deletion appears to be associated with higher tumor grade (67). However, CTNNB1 is not a tumor suppressor. The oncogenic role of CTNNB1 is through its interaction with the T-cell factor-lymphoid enhancer factor-binding protein family (64). CTNNB1 promoter methylation was not observed in esophageal adenocarcinomas (68). CTNNB1 has not been investigated previously in human endometrial cancers. Methylation of CTNNB1 was present in 16.7% of primary tumors in this series. The methylation observed could reflect a molecular change leading to inactivation of CTNNB1 or may have no effect on expression. Immunohistochemistry to assess CTNNB1 expression in endometrial cancers could shed light on the relationship between methylation and gene expression.

VDR (CYP27B) converts vitamin D to its active form. CYP27B has been implicated in cell cycle control (antiproliferation) and prodifferentiation in many different tissues (48). Decreased expression has been found in endometrial cancer cell lines (49). However, the cause of such down-regulation has not been determined. Treatment of various cancers with vitamin D has been proposed based on its ability to differentiate cells and to form glands specifically in endometrial cancer (49). A study in rats, treated with dimethylhydrazine to induce colonic adenocarcinoma, revealed a high level of methylation in the VDR promoter CpG island (69). It is noteworthy that inhibition of methylation was seen in the rats treated with dimethylhydrazine + estradiol, with a significantly lower number of malignancies in this cohort (69). Methylation seen in 1 of 24 of the tumors in our series is likely not the cause of frequent decreased VDR expression in endometrial cancer.

Methylation of the HOXA11 promoter has not been examined previously in human endometrial cancers. HOXA11 encodes a transcription factor that plays an important role in the embryologic development of the endometrium (46, 47). HOXA11 is expressed in both the epithelium and stroma in the adult uterus, and appears to be regulated by ovarian steroids (70–73). Given the role of HOXA11 in maintaining the “plastic” nature of the adult endometrium, it is possible that epigenetic changes and altered expression of HOXA11 could contribute to endometrial malignancies.

We observed a statistically significant association between HOXA11 promoter methylation in endometrial cancers and recurrence. More than 90% of primary tumors (11 of 12) that later recurred demonstrated methylation of this locus, whereas only 51% (19 of 37) of nonrecurrent primary tumors were methylated (P = 0.0167). The follow-up times for the recurrent and nonrecurrent patients were similar (Table 3). Normal peripheral blood leukocyte DNA from the endometrial cancer cases we studied did not show HOXA11 methylation, suggesting that the methylation observed may be a tumor-specific event. The mouse Hoxa11 locus was shown recently to be methylated in uterine DNA prepared from mice that had been treated with DES (74). Treatment with DES in the neonatal period results in nearly 100% penetrance of uterine adenocarcinomas by age 18 months (75). The uteri from 18-month-old DES-treated mice showed nearly 20% HOXA11 methylation (75). Taken together, our studies of human endometrial cancers and the report on DES-treated mouse uteri suggest that HOXA11 methylation may contribute to endometrial tumorigenesis.

One drawback to the study reported here is the limited spectrum of endometrial cancers evaluated. The tumors we evaluated were all from patients with stage I and II disease. The cases were matched for stage and grade, and recurrence was the primary outcome variable. They are representative of early stage endometrioid adenocarcinomas (stage 1A through 1IB) in general. The age at diagnosis, grade, and stage were similar in the recurrent and nonrecurrent cases. An analysis of additional tumor specimens should be evaluated to confirm the prognostic significance of HOXA11 methylation in endometrial cancers. Alternative methods to assess methylation, such as methylation-specific PCR, and the use of archived tissues from cases for which outcome data are already available could be used to confirm our finding that HOXA11 methylation is a prognostic indicator. Study of higher stage tumors, and/or different histological types may also be important in unraveling the relationship between methylation and outcome. Correlating methylation with expression levels will be an important first step in coming to understand how HOXA11 methylation might contribute to the cancer phenotype.

The putative THBS2 promoter region, 5′ to exon 1, contained a limited number of CpG pairs, and was not considered a true CpG island as defined by Bird (31) in 1986. There is a CpG island additionally downstream (intron and exon 1B). We were unable to devise a COBRA assay for these sequences (inadequate PCR products). Further studies on the THBS2 CpG island may be warranted.

A high Mel, and likely methylator phenotype, exists in endometrioid endometrial adenocarcinoma for the panel of genes studied. A methylator phenotype in endometrial cancer
has not been reported previously. Additional analysis in a wider range of cases will be necessary to determine whether high MeI correlates with prognosis or other clinicopathologic features. Many of the candidate genes we investigated are expected to play roles in endometrial cancer development, prognosis, and possibly in response to treatment. HOXA11 and THBS2 promoter methylation in endometrial adenocarcinoma is more common than methylation described for other genes in endometrial cancer. Regulatory functions for these candidates in embryogenesis, endometrial proliferation during the menstrual cycle, and angiogenesis strongly suggest an early role in endometrial cancer development. HOXA11 methylation appears to be a molecular event that can predict recurrence in stage I and II endometrioid endometrial adenocarcinoma. However, additional studies of HOXA11 methylation will be required to determine the prognostic significance and potential clinical utility of HOXA11 promoter methylation in endometrial cancer.

ACKNOWLEDGMENTS

We thank Christina Todd-Menke for assistance with bisulfite conversions and MLH1 promoter methylation studies, and Dr. Tim Huang for helpful discussions. We also thank Tonia Thompson for help with manuscript preparation.

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