Frequent *HOXA11* and *THBS2* Promoter Methylation, and a Methylator Phenotype in Endometrial Adenocarcinoma[1]

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ABSTRACT

Purpose: **This study was designed to determine whether there is a methylator phenotype in stage I and II endometrioid 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 endometrial cancers 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

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[3] The abbreviations used are: *CTNNB1*, β-catenin; MSI, microsatellite instability; *THBS*, thrombospondin; *CIMP*, CpG island methylator phenotype; *HOXA11*, homeobox A11; *VDR*, vitamin D receptor (25-hydroxyvitamin D3–1-α-hydroxylase); *MLH1*, mutL DNA mismatch repair gene; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; COBRA, combined bisulfite restriction analysis; MeI, methylation index.

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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* ($p16^{INK4a}$) 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). *HOXA11* 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 *HOXA11* 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 adherens 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 *HOXA11* 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 *HOXA11* 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: *HOXA11*, *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

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Table 1 Gene sequences evaluated for methylation in endometrial adenocarcinomas

Symbol	Gene	Chromosomal location	NCBI[a] accession	Amplimer location[b]	Cytosines evaluated[c]
HOXA11	Homeobox A11	7p15-p14	AF071164	3637–3791	*Taq*I 3679 *Bst*UI 3730/3732 3734/3736
THBS2	Thrombospondin-2	6q27	U79410	4811–4948	*Taq*I 4872 *Rsa*I 4895
MLH1	*Escheria coli* mutL homologue	3p21.3	U26559	601–715	*Sau*3A I 672 *Bst*UI 684/686
CTNNB1	β-catenin	3p22-p21.3	X89448	795–1088	*Bst*UI 858/860 885/887 905/907/909 922/924 960/962 *Hha*II[c] 858/860 960/962
VDR	Vitamin D receptor (25-hydroxyvitamin D3-1 α hydroxylase)	12q13.3	AB005990	302–521	*Taq*I 375 *Bst*UI 454/456
CDKN2A	Cyclin-dependent kinase inhibitor 2A ($p16^{INK4a}$)	9p21	U12818	16–273	*Bst*UI 46/48 161/163 *Taq*I 226, 247
THBS1	Thrombospondin	15q15	J04835	2158–2440	*Bst*UI 2257/2259/2261 2410/2412 *Taq*I 2291

* Refers to sequences amplified in the second round of PCR, and subjected to restriction analysis.
[a] NCBI, National Center for Biotechnology Information.
[b] Refers to locations of nucleotides (cytosines) assessed by restriction digestion.
[c] *Hha*I sites evaluated same CpG pairs as *Bst*UI digestion.

with UV illumination. The unrestricted and restricted PCR products were run side-by-side. Photoimages (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 ≥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 (32, 33), ranged from 0 to 0.43 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). Representative ex-

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Table 2 Primers and conditions for amplification of promoter region for COBRA analyses[a]

Gene	Outer primers (round 1) 5′→ 3′		Annealing temp (°C)	Inner primers (round 2) 5′→ 3′		Annealing temp (°C)
	Forward	Reverse		Forward	Reverse	
HOXA11	ggTttTTtttTttgtagTTa	ccaAAAcatccctcacccaccc	48	TTaTTtTaggggaagTaaTaga	aAAaccctAcaattAaAcacaa	55
THBS2	tgtataTTTtgaTTtggga	ttAccaacatttatctcaAAc	48	gggtgatgTTtgaggtgtgggag	caaAtccccttAAtAcacactt	55
MLH1	tttTtTaaTtTtgtgggttgTtggg	AAaAAccacaaAaAcaAAAccaa	55	TtgTTcg[b]TtaTTtagaaggatatg	tctActcctattAActAAatatttc	60
CTNNB1	ggaggataTTagggTTaTTttTT	AcacctcaAAAAaacaAActcct	48	gtggggtgTTTaggTaTTTTa	AaaActActcctcaAaccttcctc	58
CDKN2A	gtTTTtTTagaggatttgaggg	tacctAattccaattcccctAc	48	gTtggTtggtTaTTagagggtgg	ctAcaAaccctctacccacct	60
VDR	gtgTagagTaaatgtgggaTag	atAtctAAactAtccatctca	48	ggatgTTTatgaaataaggaa	AAaacccatAtaActttaaAcc	58
THBS1	gaTttttTtgagaagttTtagtgT	ccaccaAaAAAactAaaAcctca	48	ggagagaggagTTTagaTtggTTT	AAAAcAacttacctAtAtAtacc	55

[a] Capitals indicate bases that have been bisulfite converted.
[b] Because of high CpG concentration in this region, *MLH1* forward inner primer contains a CG pair.

amples of COBRA analyses to assess methylation are shown in Fig. 2.

***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 *Taq*I and *Bst*UI 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 *Taq*I and *Bst*UI 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 tumor-specific 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

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	Sample	Promoters Analyzed							MeI
		HOXA11	*THBS2*	*MLH1*	*CTNNB1*	*VDR*	*CDKN2A*	*THBS1*	
Cell Lines	KLE	■	□	□	□	□	□	□	0.14
	AN3CA	■	□	■	□	■	■	□	0.57
	ISHIKAWA	■	□	□	■	□	□	□	0.29
	RL952	■	■	□	□	□	□	□	0.29
	HECIA	■	■	□	■	■	■	□	0.71
Bloods	BL1	□	□	□	□	□	□	□	0.00
	BL2	□	□	□	□	□	□	□	0.00
Primary tumors with recurrence	1029	■	■	□	□	□	□	□	0.29
	0008	■	■	□	□	□	□	□	0.29
	1093	■	■	□	□	□	□	□	0.29
	1359	■	□	■	□	□	□	□	0.29
	1085	■	□	□	□	□	□	□	0.14
	1087	□	■	■	□	□	□	□	0.29
	0082	■	■	■	□	□	□	□	0.43
	0124	■	■	□	□	□	□	□	0.29
	1176	■	□	□	□	□	□	□	0.14
	1191	■	■	□	□	□	□	□	0.29
	0002	■	□	□	□	□	□	□	0.14
	1112	■	■	■	□	□	□	□	0.43
Primary tumors without recurrence	1257	□	□	■	□	□	□	□	0.14
	0064	■	■	■	□	□	□	□	0.43
	1110	■	■	□	□	□	□	□	0.29
	1028	□	■	□	■	□	□	□	0.29
	1319	■	■	□	□	■	□	□	0.43
	1059	■	■	□	■	□	□	□	0.43
	1072	□	□	□	□	□	□	□	0.00
	1256	□	■	□	■	□	□	□	0.29
	1271	□	□	□	□	□	□	□	0.00
	1330	■	□	■	□	□	□	□	0.29
	1247	■	■	□	■	□	□	□	0.43
	1173	□	□	■	□	□	■	□	0.29
	% TUMORS METHYLATED	70.8	62.5	33.3	16.7	4.2	4.2	0	

Fig. 1 Patterns of methylation of *HOXA11, THBS2*, *MLH1*, *CTNNB1*, *VDR*, *CDKN2A*, and *THBS1* in primary endometrial cancers and endometrial cancer cell lines. ■ represent methylated promoter regions. □ represent unmethylated loci. MeI, number of promoters methylated ÷ total number of promoters analyzed.

revealed methylation of both the *Taq*I and *Bst*UI sites in the *HOXA11* promoter, and as such, the blood DNAs were classified as unmethylated. Methylation was observed at a single CpG (*Taq*I site) in 4 of 24 of the normal bloods (data not shown). All 4 of the patients with *HOXA11 Taq*I 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 $\geq$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 *Taq*I and *Bst*UI 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.

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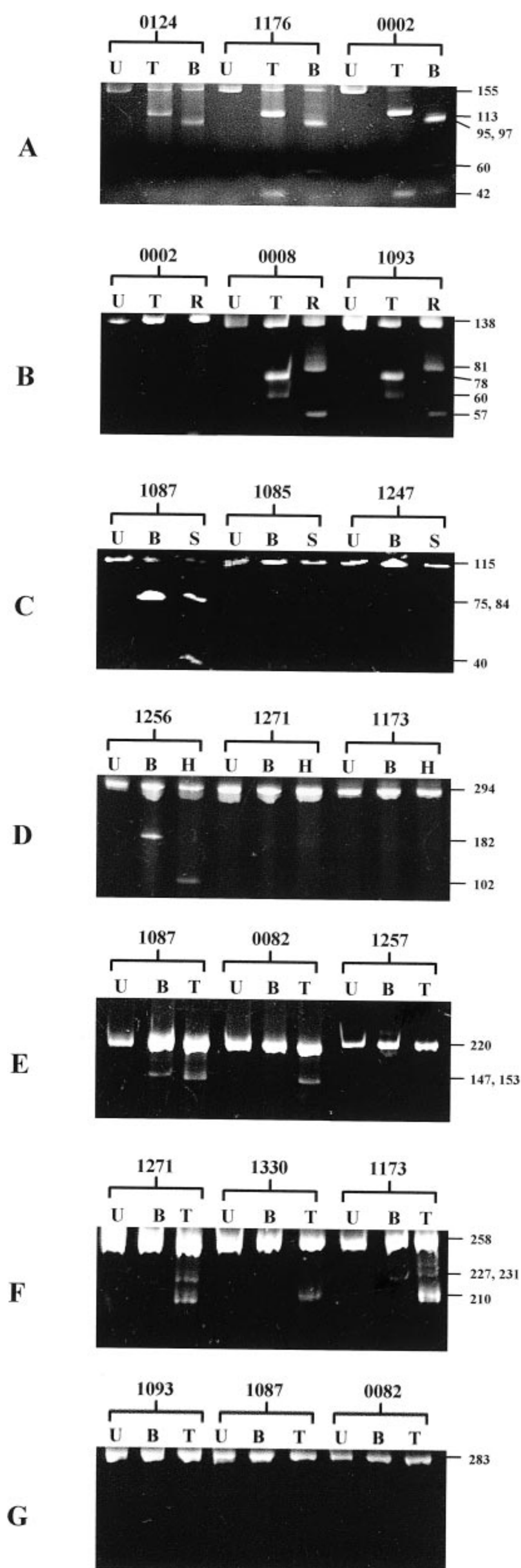

Fig. 2 COBRA methylation analysis in primary endometrial cancers. *A, HOXA11* partial methylation in all three tumors shown, with nearly full methylation in tumor 0002. *B, THBS2* partial methylation in tumors 0008 and 1093, and no methylation in tumor 0002. *C, MLH1* methylation in tumor 1087. Tumors 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 0082 demonstrates methylation at the *Taq*I 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 *Taq*I enzyme. *G, THBS1* was not methylated in any of the tumors analyzed. *T*, *Taq*I; *B*, *Bst*UI; *H*, *Hha*I; *R*, *Rsa*I; *S*, *Sau3*AI; *U*, unrestricted PCR product. Fragment sizes are indicated in bp, and tumor identifiers are shown *above* each representative gel.

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

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Table 3 *HOXA11* and *THBS2* promoter methylation in endometrial adenocarcinomas The percentage (%) methylation was estimated by taking area of peak signal densities of restricted products divided by area of unrestricted PCR product for each assay × 100. Twenty four stage I/II tumors in the original series, and an additional 25 nonrecurrent tumors were analyzed for methylation of the *HOXA11* and *THBS2* promoter regions. Cases were classified as methylated when both enzymes digested and there was ≥10% average combined methylation (shaded).

Series	Group	*HOXA11* Sample	Follow-up (months)	% Methylation		Average	*THBS2* Sample	% Methylation		Average
				Taq I	BstU I			Taq I	Rsa I	
Original series of matched recurrent and nonrecurrent cases	Recurrent	1087	40	0	0	0	1087	17	9	13
		1029	29	41	8	25	1029	22	5	14
		0008	111	72	19	46	0008	61	52	57
		1093	42	46	28	37	1093	60	34	47
		1359	23	22	7	15	1359	10	6	8
		1085	27	36	11	24	1085	8	0	4
		0082	22	22	14	18	0082	24	13	19
		0124	38	41	37	39	0124	19	5	12
		1176	49	89	82	86	1176	31	0	16
		1191	33	38	8	23	1191	18	8	13
		0002	77	99	95	97	0002	0	0	0
		1112	13	30	34	32	1112	10	11	11
	Nonrecurrent	1257	31	0	0	0	1257	0	0	0
		1072	72	0	0	0	1072	20	0	10
		1256	2	0	0	0	1256	31	26	29
		1271	37	0	0	0	1271	18	0	9
		1173	53	0	0	0	1173	27	0	14
		1028	80	6	6	6	1028	42	24	33
		1330	25	26	13	20	1330	0	23	12
		1247	47	62	43	53	1247	3	18	11
		0064	63	59	34	47	0064	20	16	18
		1110	39	27	24	26	1110	24	18	21
		1319	27	37	25	31	1319	34	13	24
		1059	74	51	7	29	1059	18	7	13
Additional primary tumors	Nonrecurrent	1185	57	0	0	0	1185	0	0	0
		1129	21	0	0	0	1129	27	2	15
		1340	25	0	0	0	1340	50	56	53
		1158	57	0	0	0	1158	25	26	26
		1109	64	0	0	0	1109	26	15	21
		1065	65	0	0	0	1065	46	71	59
		1070	69	0	0	0	1070	26	34	30
		1190	28	0	0	0	1190	14	30	22
		1145	48	0	0	0	1145	0	0	0
		1294	33	16	0	8	1294	14	3	9
		1095	66	50	0	25	1095	15	16	16
		1117	24	6	6	6	1117	13	23	18
		1114	72	23	13	18	1114	0	0	0
		1216	46	46	34	40	1216	27	22	25
		1233	40	100	53	77	1233	10	25	18
		1267	37	25	30	28	1267	0	0	0
		1270	37	73	77	75	1270	17	65	41
		1293	31	30	29	30	1293	0	0	0
		0159	85	47	29	38	0159	21	10	16
		1042	46	25	17	21	1042	28	25	27
		1138	32	37	11	24	1138	27	28	28
		1062	7	61	100	81	1062	0	0	0
		1089	37	24	19	22	1089	8	28	18
		1251	39	55	44	50	1251	11	25	18
		1314	34	19	18	19	1314	15	23	19

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,

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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 IA through IIB) 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 MeI, and likely methylator phenotype, exists in endometrioid endometrial adenocarcinoma for the panel of genes studied. A methylator phenotype in endometrial cancer

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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.

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