Short Communication

Methylated DNA Collected by Tampons—A New Tool to Detect Endometrial Cancer

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Abstract

This proof of principle study aimed to define a new and simple strategy for detection of endometrial cancer using epigenetic markers. We investigated DNA isolated from vaginal secretion collected from tampon for aberrant methylation of five genes (*CDH13*, *HSPA2*, *MLH1*, *RASSF1A*, and *SOCS2*) using MethyLight in 15 patients with endometrial cancer and 109 patients without endometrial cancer. All endometrial cancer patients revealed three or more methylated genes, whereas 91% (99 of 109) of the patients without endometrial cancer had no or fewer than three genes methylated in their vaginal secretion. The methods developed in this study provide the basis for a prospective clinical trial to screen asymptomatic women who are at high risk for endometrial cancer. (Cancer Epidemiol Biomarkers Prev 2004;13(5):882–8)

Introduction

Endometrial cancer is one of the most common genital cancers in women worldwide. The highest incidence rates are observed in western Europe and North America (1). The well-known risk factors for endometrial cancer include obesity, type 2 diabetes mellitus, and hypertension. Additionally, anovulation and long-term use of unopposed estrogens for hormone replacement therapy increase the risk for endometrial cancer. Genetic causes of endometrial cancer are uncommon, although there is an association with hereditary nonpolyposis colon cancer syndrome, in which the individual risk rises to a cumulative incidence of 40% by age 70 years (2). In 2001, the American Cancer Society concluded that there was insufficient evidence to recommend screening for endometrial cancer for women at average risk or increased risk due to history of unopposed estrogen therapy, nulliparity, infertility or failure to ovulate, obesity, diabetes, or hypertension (3). Studies examining endometrial carcinoma screening methods for asymptomatic postmenopausal women have used ultrasound-determined endometrial thickness as an indication of risk. Transvaginal ultrasonography compared with endometrial biopsy for the detection of endometrial disease had a positive predictive value of only 9% for detecting any abnormality, with 90% sensitivity and 48% specificity (4). There is a need for a sensitive and specific screening test for high-risk women. It has been shown that genetic abnormalities can be used to detect endometrial cancer (5). Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasias (6). It has been increasingly recognized over the past 4–5 years that the CpG islands of a large number of genes, which are unmethylated in normal tissue, are methylated to varying degrees in multiple types of human cancer (6, 7). Aberrant methylation of CpG islands within the promoter regions of several genes such as *E-cadherin*, *adenomatous polyposis coli* (*APC*), *MLH1*, *p16*, *estrogen receptor*, *progesterone receptor*, and *PTEN* (*MMAC1*) has been identified in endometrial cancer tissue (8–14). Up to now, no investigations have been undertaken to assess the methylation status of DNA obtained from cervicovaginal secretion from endometrial cancer patients.

Our proof of principle study was performed to determine whether it is possible to detect endometrial cancer by analyzing methylated DNA in cervicovaginal secretion.

Materials and Methods

Patients and Samples. One hundred twenty-four patients were recruited for this study: 15 patients had endometrial cancer, while the no endometrial cancer group contained 5 patients with invasive cervical cancer, 35 patients with cervical intraepithelial neoplasia

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Note: H. Fiegl, C. Gattringer, and A. Widschwendter contributed equally to this work.

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(CIN I, 3 cases; CIN II, 19 cases; CIN III, 13 cases), and 69 patients with benign disease of the uterus. Histology of the endometrial cancers was adenocarcinoma in 12 cases, adenosquamous carcinoma in 2 cases, and malignant mixed mullerian tumor in 1 case. Most of the carcinomas (10 cases) were well differentiated (tumor grade 1). Only four carcinomas had tumor grade 2. All FIGO stages were represented in the endometrial cancer group (FIGO I, 8 patients; FIGO II, 2 patients; FIGO III, 3 patients; FIGO IV, 1 patient; unknown FIGO stage, 1 patient). Sample collection was done between January 1, 2003 and May 31, 2003 at the Department of Obstetrics and Gynecology, Innsbruck University Hospital, Austria. All patients who were scheduled to undergo surgery of the uterus on the next day including a histological diagnosis were invited to attend the study. Samples and clinical data were collected after informed consent was obtained. To ensure standardized sample collection, a tampon was inserted in the patient by a physician after speculum examination and retained intravaginal for 30 min. Preparation of the samples is shown in Fig. 1.

DNA Isolation and Methylation Analysis. Genomic DNA from samples was isolated using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with some modifications for multiple loading of the DNA extraction columns to gain a sufficient amount of DNA. Sodium bisulfite-treated genomic DNA was analyzed by means of MethyLight, a fluorescence-based, real-time PCR assay, as described previously (15, 16). Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set, β-actin (*ACTB*), to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *Sss*I (New England Biolabs, Frankfurt, Germany)-treated human WBC DNA (heavily methylated). The percentage of fully methylated reference (PMR) molecules at a specific locus was calculated by dividing the *GENE*:*ACTB* ratio of a sample by the *GENE*:*ACTB* ratio of *Sss*I-treated WBC DNA and multiplying by 100. PMR indicates this measurement. A gene was deemed methylated if the PMR value was >0. Primer and probes specific for methylated DNA and used for MethyLight reactions are listed in Table 1.

Statistical Analysis. Associations between categorical variables were tested using the χ^2 test. Differences in median of age were examined with the Mann-Whitney *U* test or, between more than two groups, with the Kruskal-Wallis test. Due to a significant age difference between endometrial cancer patients and the no endometrial cancer group, an age-matched group of the no endometrial cancer group with a matching ratio 1:2 was randomly selected. The computation of the matching group was done using MATLAB R12 (http://www.mathworks.com). For determination of diagnostic accuracy, a nonparametric receiver operating curve analysis with linear interpolation was performed. $P > 0.05$ was considered statistically significant. All statistical calculations were performed using SPSS version 11.0 for Windows.

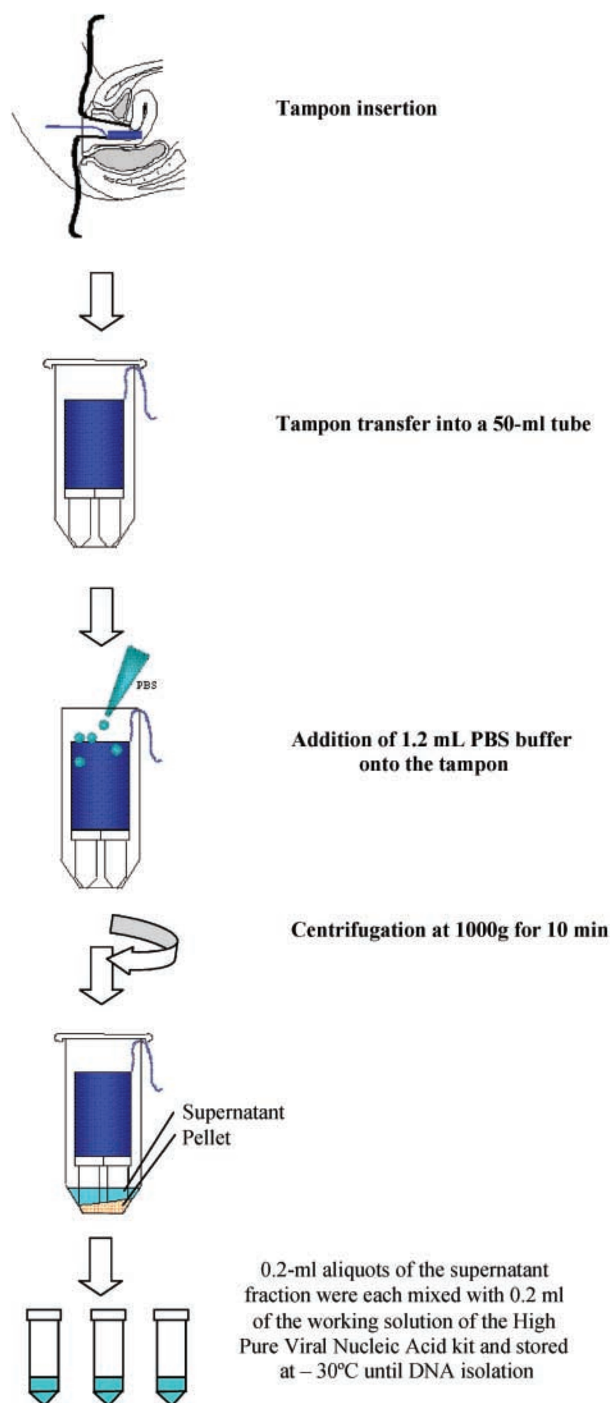

Figure 1. Flowchart of procedures for tampon insertion and sample preparation.

Results

Aberrant methylation of 38 genes in DNA obtained from vaginal secretion from the first five patients with endometrial cancer and the first four patients with benign disease was analyzed to determine appropriate genes for further study. The most appropriate genes for our further

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Table 1. Primer and probes specific for methylated DNA and used for MethyLight reactions

HUGO gene nomenclature	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
ACTB	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	6FAM-ACCACCACCCAACACACAATAACAAACACA-BHQ-1
APC	GAACCAAAACGCTCCCCAT	TTATATGTCGGTTACGTGCGTTTATAT	6FAM-CCCGTCGAAAACCCGCCGATTA-BHQ-1
ARHI	GCGTAAGCGGAATTTATGTTTGT	CCGCGATTTTATATTCCGACTT	6FAM-CGCACAAAAACGAAATACGAAAACGCAAA-BHQ-1
BLT1	GCGTTGGTTTTATCGGAAGG	AAACCGTAATTCCCGCTCG	6FAM-GACTCCGCCCAACTTCGCCAAAA-BHQ-1
BRCA1	GAGAGGTTGTTGTTTAGCGGTAGTT	CGCGCAATCGCAATTTTAAT	6FAM-CCGCGCTTTTCCGTTACCACGA-BHQ-1
CALCA	GTTTTGGAAGTATGAGGGTGACG	TTCCCGCCGCTATAAATCG	6FAM-ATTCCGCCAATACACAACAACCAATAAACG-BHQ-1
CDH1	AATTTTAGGTTAGAGGGTTATCGCGT	TCCCCAAAACGAAACTAACGAC	6FAM-CGCCCACCCGACCTCGCAT-BHQ-1
CDH13	AATTTCGTTCGTTTTGTGCGT	CTACCCGTACCGAACGATCC	6FAM-AACGCAAAACGCGCCCGACA-BHQ-1
CDKN2A	TGGAGTTTTCGGTTGATTGGTT	AACAACGCCCGCACCTCCT	6FAM-ACCCGACCCCGAACCGCG-BHQ-1
CYP1B1	GTGCGTTTGGACGGGAGTT	AACGCGACCTAACAAAACGAA	6FAM-CGCCCGCACACCAAACCGCTT-BHQ-1
DAPK1	TCGTCGTCGTTTCGGTTAGTT	TCCCTCCGAAACGCTATCG	6FAM-CGACCATAAACGCCAACGCCG-BHQ-1
ESR1	GGCGTTCGTTTTGGGATTG	GCCGACACGCGAACTCTAA	6FAM-CGATAAAACCGAACGACCCGACGA-BHQ-1
ESR2	TTTGAAATTTGTAGGGCGAAGAGTAG	ACCCGTCGCAACTCGAATAA	6FAM-CCGACCCAACGCTCGCCG-BHQ-1
FGF18	ATCTCCTCCTCCGCGTCTCT	TCGCGCGTAGAAAACGTTT	6FAM-CGACCGTACGCATCGCCGC-BHQ-1
GSTM3	GCGCGAACGCCCTAACT	AACGTCGGTATTAGTCGCGTTT	6FAM-CCCCGTTCTCCGTCCCTTACCTCC-BHQ-1
GSTP1	GTCGGCGTCGTGATTTAGTATTG	AAACTACGACGACGAAACTCCAA	6FAM-AAACCTCGCGACCTCCGAACCTTATAAAA-BHQ-1
HIC1	GTTAGGCGGTTAGGGCGTC	CCGAACGCCTCCATCGTAT	6FAM-CAACATCGTCTACCCAACACACTCTCCTACG-BHQ-1
HLA-G	CACCCCCATATACGCGCTAA	GGTCGTTACGTTTCGGGTAGTTTA	6FAM-CGCGCTCACACGCTCAAAAACCT-BHQ-1
HSD17B4	TATCGTTGAGGTTCGACGGG	TCCAACCTTCGCATACTCACC	6FAM-CCCGCGCCGATAACCAATACCA-BHQ-1
HSPA2	CACGAACACTACCAACAACTCAACT	GGGAGCGGATTGGGTTTG	6FAM-CCGCGCCCAATTCCCGATTCT-BHQ-1
IGFBP2	CTCGCGCCGACAAATAAATAC	CGGGAAGAGTAGGGAATTTTTAGAGT	6FAM-ACGCCCGCTCGCCCACCT-BHQ-1
MGMT	GCGTTTCGACGTTCGTAGGT	CACTCTTCCGAAAACGAAACG	6FAM-CGCAAACGATACGCACCGCGA-BHQ-1
MLH1	AGGAAGAGCGGATAGCGATTT	TCTTCGTCCCTCCCTAAAACG	6FAM-CCCGCTACCTAAAAAAATATACGCTTACGCG-BHQ-1
MLLT7	CCTCACGATACCTCCCCTCAA	TTAGGGATTAGCGTTTTGGGATT	6FAM-AAACACATTCCTACCAATCTTCAAAAAATCGCG-BHQ-1
MT3	CGATAAACGAACTTCTCCAAACAA	GCGCGGTGCGTAGGG	6FAM-AAACGCGCGACTTAACTAATAACAACAAATAACGA-BHQ-1
MYOD1	GAGCGCGCGTAGTTAGCG	TCCGACACGCCCTTTCC	6FAM-CTCCAACACCCGACTACTATATCCGCGAAA-BHQ-1
PGR	TTATAATTCGAGGCGGTTAGTGTTT	TCGAACTTCTACTAACTCCGTACTACGA	6FAM-ATCATCTCCGAAAATCTCAAATCCCAATAATACG-BHQ-1
PPP1R13B	CCTCACCCACCGACATCATC	TCGGAGCGGTGGGTATAGTTC	6FAM-AAAAATCCGCGACGCCCTCGA-BHQ-1
PTGS2	CGGAAGCGTTCGGGTAAAG	AATTCCACCGCCCCAAAC	6FAM-TTTCCGCCAAATATCTTTTCTTCTTCGCA-BHQ-1
RASSF1A	ATTGAGTTGCGGGAGTTGGT	ACACGCTCCAACCGAATACG	6FAM-CCCTTCCCAACGCGCCCA-BHQ-1
SOCS1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA	6FAM-ACAATTCCGCTAACGACTATCGCGCA-BHQ-1
SOCS2	TCCCTTCCCCGCCATT	TTGTTTTTGTCGCGGTGATTT	6FAM-CCGAAAAACTCAAAACACCGCAAAATCATBHQ-1
SYK	GGGCGCGATATTGGGAG	GCGACTCTTCCTCATTTTAAACAAC	6FAM-CCTTAACGCGCCCGAACAAACG-BHQ-1
TERT	GGATTCGCGGGTATAGACGTT	CGAAATCCGCGCGAAA	6FAM-CCCAATCCCTCCGCCACGTAAAA-BHQ-1
TFF1	TAAGGTTACGGTGGTTATTTCGTGA	ACCTTAATCCAAATCCTACTCATATCTAAAA	6FAM-CCCTCCCGCCAAAATAAATACTATACTCACTACAAAA-BHQ-1
TIMP3	GCGTCGGAGGTTAAGGTTGTT	CTCTCCAAAATTACCGTACGCG	6FAM-AACTCGCTCGCCCGCCGAA-BHQ-1

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Table 1. Primer and probes specific for methylated DNA and used for MethyLight reactions (Cont'd)

HUGO gene nomenclature	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
TITF1	CGAAATAAACCGAATCCTCC TTAA	TGTTTTGTTGTTTTAGCGTTTACGT	6FAM-CTCGCGTTTATTTTAACCCG ACGCCA-BHQ-1
TP53BP2	ACCCCCTAACGCGACTTTATC	GTTCGATTCGGGATTAGTTGGT	6FAM-CGCTCGTAACGATCGAAACT CCCTCCT-BHQ-1
TWIST	GTAGCGCGGCGAACGT	AAACGCAACGAATCATAACCAAC	6FAM-CCAACGCACCCAATCGCTAAA CGA-BHQ-1

analyses were determined to be those that revealed the greatest difference in PMR values between patients with benign disease of the uterus and endometrial cancer patients (Fig. 2). Five genes (*i.e.*, *RASSF1A*, *hMLH1*, *CDH13*, *HSPA2*, and *SOCS2*) were selected for further analysis. DNA methylation in three or more of these five genes was observed in cervicovaginal secretion of all five patients with endometrial cancer, whereas all four patients without endometrial cancer showed no or fewer than three genes to be methylated. We therefore

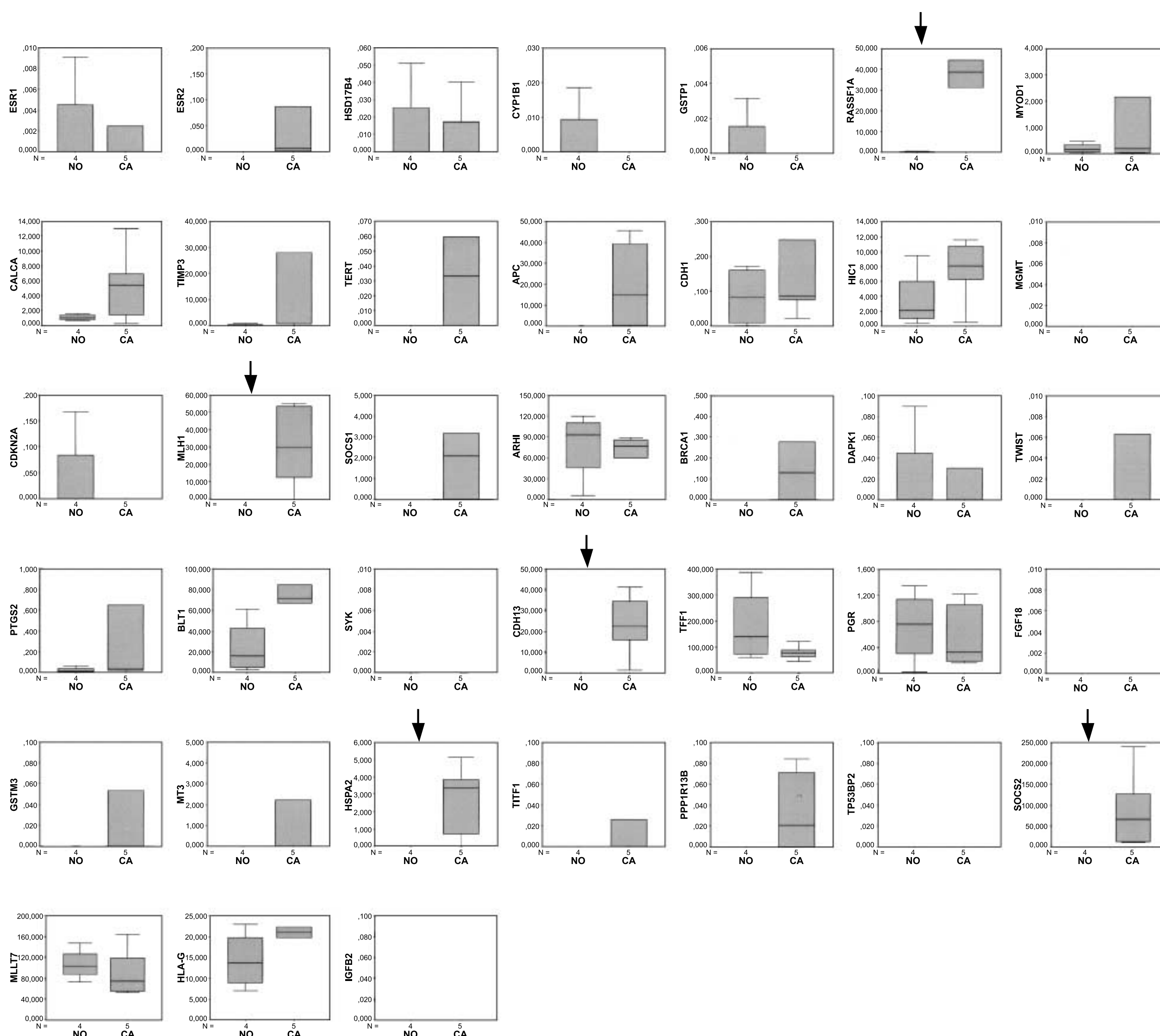

Figure 2. PMR values of 38 genes in patients without endometrial cancer (n = 4) and with endometrial cancer (n = 5). *Arrows,* genes with the greatest difference in PMR values.

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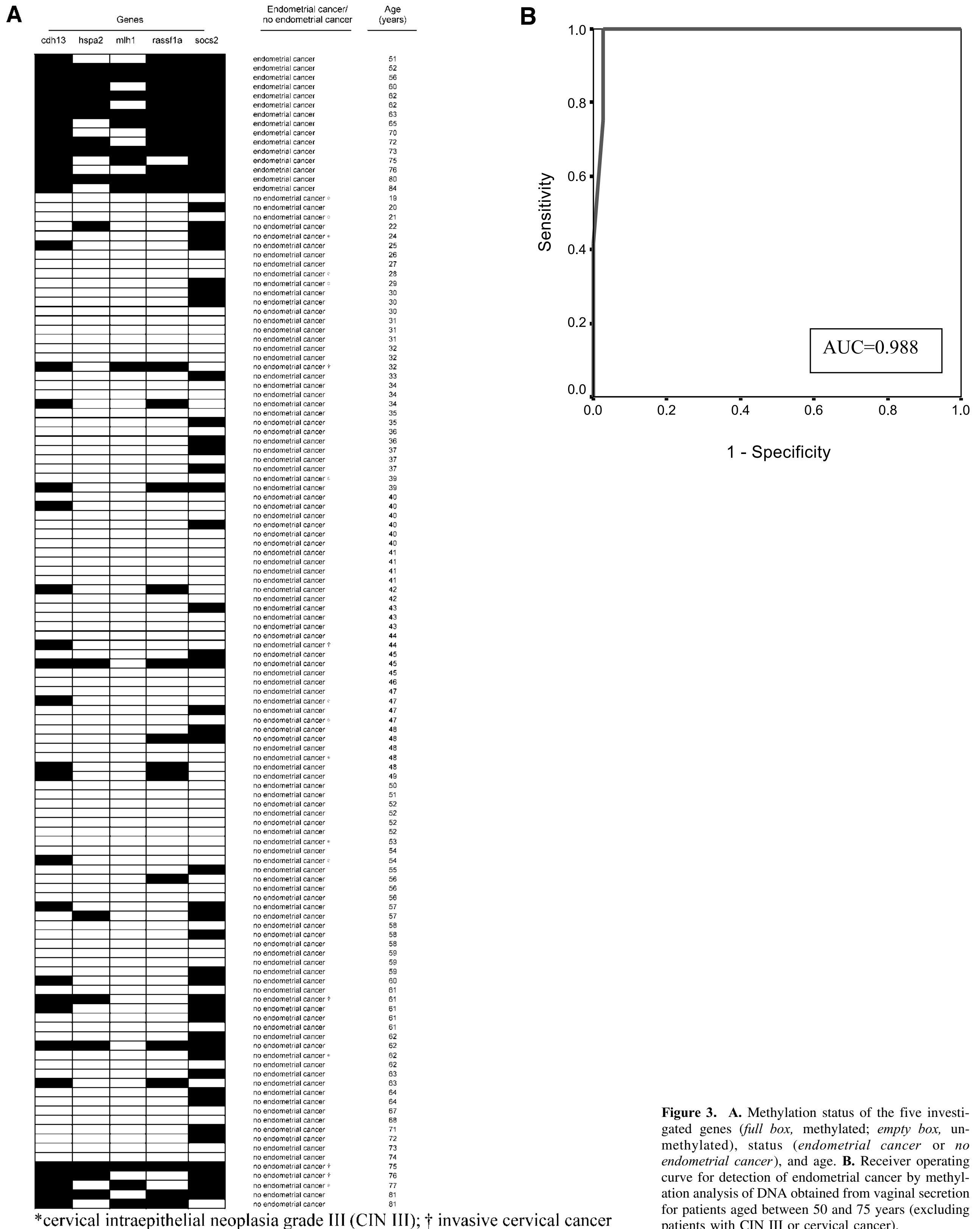

*cervical intraepithelial neoplasia grade III (CIN III); † invasive cervical cancer

Figure 3. **A.** Methylation status of the five investigated genes (*full box,* methylated; *empty box,* unmethylated), status (*endometrial cancer* or *no endometrial cancer*), and age. **B.** Receiver operating curve for detection of endometrial cancer by methylation analysis of DNA obtained from vaginal secretion for patients aged between 50 and 75 years (excluding patients with CIN III or cervical cancer).

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determined the cutoff value between no endometrial cancer and endometrial cancer as methylation positive in three or more of the five investigated genes.

The overwhelming majority of the patients without endometrial cancer (99 of 109) revealed no or fewer than three genes methylated, whereas all of the 15 endometrial cancer patients had three or more genes methylated in their vaginal secretion ($P < 0.001$, χ^2 test; Fig. 3A). Histological examination of the 10 patients in the no endometrial cancer group with three or more genes methylated revealed invasive cervical cancer (four cases), CIN III (one case), endometrium polyp (four cases), and fibroids (one case). Samples were collected after primary surgery (curettage, punch biopsy of the cervix, or hysteroscopic operation) and before secondary surgery (hysterectomy) in 16 of 124 patients: 9 of 16 patients had endometrial cancer, 3 of 16 patients had CIN III, and 4 of 16 patients had benign disease of the endometrium. All nine endometrial cancer patients had a diagnosis of residual cancer at the time of secondary surgery. All nine endometrial cancer patients had three or more genes methylated, the three CIN III patients revealed no methylated genes, and one of the patients with benign disease showed one gene to be methylated. Within the group of patients from whom the vaginal secretion was collected prior to any surgery, one patient presented due to sonographically detected serometra with complete stenosis of the cervicouterine canal. Even this patient showed methylation of three of the five tested genes. DNA methylation of the five genes identified seems to increase with age although statistically not significant (data not shown). Using all 15 endometrial cancer cases and 109 controls, the area under the receiver operating curve was 0.973 (data not shown). After exclusion of the nine pilot cases (five endometrial cancer patients and four patients without endometrial cancer), which were initially used to determine appropriate genes for further analyses, the area under the receiver operating curve was 0.963. To rule out the possibility that abnormal DNA methylation is merely a surrogate for age rather than a cancer-specific marker, we randomly age matched two nonendometrial cancer controls for each endometrial cancer case. Investigation of DNA methylation in the cervicovaginal secretion of these 45 patients was still able to discriminate between endometrial cancer and patients without endometrial cancer ($P < 0.001$, χ^2 test) with a sensitivity of 100% and a specificity of 80%.

When analyzing all patients aged between 50 and 75 years and excluding patients with CIN III or cervical cancer, sensitivity was 100% and specificity rose to 97.2% (Fig. 3B). In this group, only 1 of 35 samples was false positive. The same sensitivity and specificity values were obtained after exclusion of the pilot cases from these analyses.

Discussion

Previous studies have described the importance of DNA methylation in human cancers. Recently, aberrant methylation of various genes has been identified in endometrial cancer tissue (8–14). Methylated DNA has been investigated as a possible screening marker for neoplastic disease in several body fluids (17). However, up to now, no investigations have been undertaken to assess the methylation status of DNA obtained from cervicovaginal secretion from endometrial cancer patients.

In our study, all endometrial cancer patients revealed three or more of the five investigated genes methylated, whereas 99 of 109 patients without endometrial cancer had no or fewer than three genes methylated. Four of 10 patients in the no endometrial cancer group with three or more genes methylated had invasive cervical cancer. These cases indicate that some cervical cancer patients can also be identified with this assay.

In some cases (16 of 124), samples were collected after primary surgery and before secondary surgery. All endometrial cancer patients in this group had three or more genes methylated. These results demonstrate that aberrant methylation analysis can detect endometrial cancer even after primary surgery.

As endometrial cancer is more prevalent in older women and abnormal DNA methylation in nonmalignant tissues seems to increase with age (18), we especially addressed this problem within this project. Comparison of DNA methylation in the cervicovaginal secretion of endometrial cancer patients and age-matched nonendometrial cancer controls revealed still highly significant differences between these two groups.

Endometrial cancer occurs in almost all cases after menopause. Therefore, we analyzed all patients aged between 50 and 75 years and excluded patients with CIN III or invasive cervical cancer. These patients represent the group that will benefit from an endometrial cancer screening assay. In these group, the sensitivity and specificity to detect patients with endometrial cancer was 100% and 97.2%, respectively.

The methods developed in this study, involving tampons to collect cervicovaginal secretion, DNA isolation, and a high-throughput method to analyze DNA methylation, provide the basis for a prospective clinical trial to screen asymptomatic women who are at high risk for endometrial cancer.

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