Detection of Hypermethylated Genes in Women With and Without Cervical Neoplasia

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Background: **DNA methylation changes are an early event in carcinogenesis and are often present in the precursor lesions of various cancers. We examined whether DNA methylation changes might be used as markers of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (ICC).** ***Methods:*** **We used methylation-specific polymerase chain reaction (PCR) to analyze promoter hypermethylation of 20 genes, selected on the basis of their role in cervical cancer, in 319 exfoliated cell samples and matched tissue biopsy specimens collected during two studies of Senegalese women with increasingly severe CIN and ICC (histology negative/atypical squamous cells of undetermined significance [ASCUS] = 142, CIN-1 = 39, CIN-2 = 23, CIN-3/carcinoma in situ [CIS] = 23, ICC = 92). Logic regression was used to determine the best set of candidate genes to use as disease markers. All statistical tests were two-sided.** ***Results:*** **Similar promoter methylation patterns were seen in genes from exfoliated cell samples and corresponding biopsy specimens. For four genes (CDH13, DAPK1, RARB, and TWIST1), the frequency of hypermethylation increased statistically significantly with increasing severity of neoplasia present in the cervical biopsy (*P*<.001 for each). By using logic regression, we determined that the best panel of hypermethylated genes included DAPK1, RARB, or TWIST1. At least one of the three genes was hypermethylated in 57% of samples with CIN-3/CIS and in 74% of samples with ICC but in only 5% of samples with CIN-1 or less. The estimated specificity of the three-gene panel was 95%, and its sensitivity was 74% (95% confidence interval [CI] = 73% to 75%) for ICC and 52% (95% CI = 49% to 55%) for CIN-3/CIS. By extrapolation, we estimated that, among Senegalese women presenting to community-based clinics, detection of the DAPK1, RARB, or TWIST1 hypermethylated gene would reveal histologically confirmed CIN-3 or worse with a sensitivity of 60% (95% CI = 57% to 63%) and a specificity of 95% (95% CI = 94% to 95%).** ***Conclusions:*** **Aberrant promoter methylation analysis on exfoliated cell samples is a potential diagnostic tool for cervical cancer screening that potentially may be used alone or in conjunction with cytology and/or human papillomavirus testing. [J Natl Cancer Inst 2005;97:273–82]**

In the United States and other developed countries, the incidence of invasive cervical cancer (ICC) has decreased dramatically as a result of the identification and treatment of women with cervical intraepithelial neoplasia grade 3 (CIN-3) or carcinoma in situ (CIS), the immediate precursor lesion of ICC *(1)*. Identification of women with CIN-3/CIS is based on annual cytology screening; all women with cytologic abnormalities are referred for additional testing by repeat cytology or colposcopy and biopsy to identify CIN-3 histologically. Referral of all women with abnormal cytology for additional testing is deemed necessary because a single cytologic smear has low sensitivity (30%-60%) but high specificity (95–98%) for detecting CIN-3 *(2–5)*. In our previous study *(6)* we reported that referral on the basis of a thin-layer Pap showing ASCUS or worse provided a sensitivity of 50% and specificity of 86% for women at least 30 years old. More recently, testing for oncogenic human papillomaviruses (HPVs), which are now recognized as central to and required for development of ICC *(7,8)*, has been introduced to aid in the triage of women with atypical squamous cells of undetermined significance (ASCUS). However, the large number of women requiring additional testing and the inability of cytology or HPV testing to identify the subset of women with ASCUS or CIN of any grade who are at highest risk of further progression has made cytology-based cervical cancer control efforts increasingly costly and burdensome to the health-care system. Furthermore, the high cost and infrastructure requirements (i.e., the availability of and need for highly trained laboratory and clinical personnel) have made morphology-based screening modalities impractical in most resource-poor settings.

The need for new approaches to cervical cancer screening has led several groups to advocate primary screening based on the detection of high-risk (i.e., oncogenic) HPV types *(9)*. The use of HPV testing to identify women with, or who are at risk of developing, CIN-3/CIS is theoretically appealing. However, our study *(6)*, and others *(10,11)*, showed that, when used for primary screening, HPV testing has high sensitivity (68%–90%), but low specificity (72%–90%) for the identification of women with CIN-3/CIS, making it of little interest for primary screening for women with CIN-3/CIS or ICC *(6,10,11)*.

An alternative strategy for primary cervical cancer screening is based on the fact that, although infection with oncogenic HPVs is required for development of cervical cancer *(12)*, other molecular changes, with subsequent development of alterations in the function of gene products regulating oncogenesis, tumor suppression, DNA repair, apoptosis, metastasis and invasion, are necessary for developing a malignant phenotype *(13)*. Such alterations can result from DNA mutations or deletions or from epigenetic alterations—changes in gene expression that are not mediated by a change in the

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nucleotide sequence—such as DNA promoter hypermethylation *(14,15)*. DNA methylation refers to the addition of a methyl group to the cytosine ring of a cytosine that precedes a guanosine (referred to as CpG dinucleotides) to form methylcytosine (5-methylcytosine). In normal cells, DNA methylation plays a role in maintaining genome stability and in regulating gene expression *(16–18)*. Global hypomethylation and hypermethylation of clusters of CpG dinucleotides (referred to CpG islands) present in the promoter region of multiple genes have been associated with malignancy *(19,20)*. Hypermethylation in a promoter region is associated with "gene silencing," i.e., inhibiting expression of a gene that is normally expressed in the absence of methylation. Studies with animals and humans have demonstrated that these epigenetic methylation changes are an early event in carcinogenesis and are often present in the precursor lesions of a variety of cancers *(21–25)*. Such changes might therefore be used as markers of cervical neoplasia, either alone or in conjunction with cytology and/or HPV testing.

At present, although there is some evidence that increased rates of hypermethylation of various genes may be associated with cervical cancer *(26,27)*, few, if any, data are available regarding the sensitivity and specificity of the detection of hypermethylated genes for the identification of women with biopsy-confirmed ICC and/or various grades of cervical dysplasia. Furthermore, little is known regarding the ability to detect DNA hypermethylation in exfoliated cells versus cells in biopsy samples. The goal of the present study was to assess whether detection of hypermethylated genes in exfoliated cell samples might be used for a screening assay to identify women with ICC or CIN-3/CIS. We characterized the DNA methylation profile of 20 genes (see Appendix), including those known to be involved in cell cycle control and tissue differentiation regulation (CDKN2A, CDKN2B, CCND2, RASSF1, RARB, TWIST1, SYK, HIC1, VHL, PRDM2, and SFN), maintenance of genetic stability including DNA repair (MLH1 and MGMT), detoxification (FHIT and GSTP1), apoptosis (ASC and DAPK1), and invasion and metastasis (APC, CDH1, and CDH13). DNA hypermethylation was evaluated in exfoliated cell samples and in cervical biopsy tissues collected on the same day from a subset of women with and without cervical neoplasia or invasive cancer who were participating in a cytology screening study *(28)*. We then constructed a panel of candidate hypermethylated genes with optimal sensitivity and specificity for CIN-3/CIS or ICC and modeled the performance of this candidate "hypermethylated gene" panel in the original screening population.

Materials and Methods

Study Population

Between January 1998 and August 2000, we carried out a study whose aim was to determine the frequency of dysplasia and HPV infection in women, aged 35 years or older, presenting to community health clinics in Dakar, Senegal, for unrelated problems. The study enrolled 2609 women consecutively.

The study design and methods, participant demographic characteristics, HPV prevalence, and associations between cervical neoplasia and detection of specific HPV types and HPV variants in this study population of previously unscreened women have been reported *(28)*. According to the study protocol, all women with CIN or ICC, and those who were HPV-positive/CIN-negative, and a sample of women who were negative for both CIN and HPV were referred for colposcopy and biopsy. Cytology and HPV results from women enrolled in this study were available from 2276 (87%) women. Of the remaining 333 women, 252 (10%) had inadequate samples for cytologic evaluation, 47 (2%) had sufficient cytology but insufficient HPV samples, and 34 (1%) had missing data on screening cytology and/or HPV results.

Overall, 807 women, including 74 (83%) of 89 women with CIN-2, CIN-3/CIS or ICC, 53 (73%) of 73 women with CIN-1, 162 (65%) of 251 women with ASCUS, 98 (41%) of 239 women with negative cytology but a positive HPV test, and 325 (20%) of 1653 women with negative cytology and a negative HPV test, underwent colposcopy and biopsy. In addition, colposcopy and biopsy were performed on 82 (32%) of 252 women with unsatisfactory cytology specimens and 13 (72%) of 18 women with other cervical pathology not otherwise classified.

To ensure that the present study evaluating gene hypermethylation included a representative sample of invasive cancers, in addition to examining biopsy specimens from the "screening" study described above, we also used biopsy tissues and exfoliated cell samples collected from 391 additional women who had been referred to the University of Dakar Tumor Institute from the community health clinics in the Dakar region because of physical examination findings and/or symptoms suggestive of cervical cancer. Biopsy tissue samples and exfoliated cells from the same patient were collected on the same day. All study procedures were approved by the institutional review boards of the University of Washington (Seattle, WA) and University of Dakar (Dakar, Senegal).

To derive a panel of hypermethylated genes that were sensitive and specific for CIS/ICC, we selected a sample of 319 women, representing the full spectrum of cervical pathology (i.e., samples with and without various degrees of CIN and ICC) from among the 1198 women who had both biopsy and exfoliated cell samples available for study. This selected sample included 211 asymptomatic women from the screening study and 108 symptomatic women referred with presumed cervical cancer. Overall, 142 samples without detectable CIN on same-day cervical biopsy, 39 samples with CIN-1, 23 samples with CIN-2, 23 samples with CIN-3/CIS, and 92 samples with ICC were evaluated for detection of hypermethylated genes. Most samples from women without invasive cancer (204 [90%] of 227) were from the screening population, whereas most cases of ICC (85 [92%] of 92) were from the women presenting with presumed cancer.

Cytology and Histology Methods

Methods for collection and examination of cervical cytology and biopsy specimens have been previously described *(28)*. Briefly, biopsy material was divided into two pieces; one piece was placed in STM media (Digene Corporation, Gaithersburg, MD) for molecular assays, and the second piece was placed into formalin for histopathologic examination. Biopsy findings were interpreted by the pathologist (NBK) as negative, reactive a typical changes, CIN-1, CIN-2, CIN-3/CIS, or ICC according to World Health Organization criteria. Two exfoliated cell samples were placed into PreservCyt (Cytyc Corporation, Marlborough, MA) and STM media. The exfoliated cells stored in PreservCyt were classified as negative, ASCUS, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, CIS, or ICC according to the Bethesda criteria *(29)*. All samples were interpreted by the study pathologist (NBK) without knowledge of clinical or other laboratory findings.

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Genomic DNA Isolation and Assessment of DNA Hypermethylation

Genomic DNA was isolated from both exfoliated cells and biopsy material collected in STM media using the QIAamp DNA mini kit (Qiagen, Valencia, CA). Samples were first digested with 0.1 mg/mL protease K at 37 °C for 2 hours. DNA was extracted from 200 µL of the protease K-digested samples following the manufacturer's recommended protocol. DNA concentrations were measured using a fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

For the DNA methylation studies, 1 µg of genomic DNA was processed and modified with sodium bisulfite using the Intergen CpGenome DNA modification kit (Intergen, Norcross, GA). Briefly, genomic DNA was modified by sodium bisulfite, desulfonated with sodium hydroxide, and then purified and resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 7.5). Two sets of primers were constructed for each of the 20 genes examined (SFN, HIC1, CDH1, CDH13, APC, DAPK1, TWIST1, RARB, SYK, ASC, CDKN2A, FHIT, MGMT, CCND2, PRDM2, CDKN2B, RASSF1, GSTP1, MLH1, and VHL): U primers were designed to amplify unmethylated DNA, and M primers were designed to amplify methylated DNA (see Appendix for primer sequences and annealing temperatures). Hot start polymerase chain reaction (PCR) was performed with ampliTag Gold (Applied Biosystems, Foster City, CA, manufactured by Roche, Branchburg, NJ) using the following parameters: 95 °C for 10 minutes; 95 °C for 45 seconds, Ta°C (i.e., annealing temperature) for 45 seconds, 72 °C for 1 minute, for 35 cycles; and a final step at 72°C for 10 minutes. PCR products were separated by electrophoresis through either 5% polyacrylamide gels (for PCR products smaller than 100 bp) or 2% agarose gels containing ethidium bromide (for PCR products larger than 100 bp). Human sperm DNA and in vitro methylated (using SssI CpG methylase) human sperm DNA were used as U and M DNA controls, respectively. Methylation-specific PCR was performed twice on all specimens when adequate DNA was available, with a third repeat assay performed if discrepant results were obtained from the first two assays. Methylation of a specific gene was considered to be present if both the specimen and the M control DNA but not the U control DNA were amplified by M primers after sodium bisulfite modification. Similarly, methylation was considered to be absent if the M control DNA but neither the sample nor U control DNA was amplified by M primers. For each gene, if the M control DNA did not amplify or the U control DNA did amplify, then the test was considered invalid. The PCR results were read independently by two people (HL and a person not associated with the study) who were blinded to the histology or cytology results.

Statistical Analysis

To construct a panel of hypermethylated genes with optimal sensitivity and specificity for detecting CIN-3/CIS or ICC, we first assessed the methylation profile of 20 genes among women with various degrees of biopsy-confirmed cervical neoplasia. The Mantel–Haenszel chi-square test for trend was used to assess the statistical significance of the trend in the proportion of samples in which methylation was detected with increasing grade of histologic abnormality. Bonferroni corrections were made to adjust for multiple comparisons among the 20 genes, resulting in a cutoff P value of 0.001 to determine statistical significance.

To identify combinations of genes that provided the highest potential sensitivity and specificity for ICC and CIN-3/CIS, we started with the single gene providing the highest sensitivity for ICC (and then CIN-3/CIS) and then selected additional genes providing the largest increase in sensitivity without unreasonable loss of specificity. Logic regression was used to determine the best model, i.e., the model having the lowest score, for each desired number of genes included *(30)*. The score was defined as the average of the proportion of the classification mistakes among the case samples (1–sensitivity) and the control samples (1–specificity). The sensitivity and the specificity of the logic rule were calculated, and a leave-one-out cross-validation was performed to estimate how well the model would fit an independent sample. After repeating this procedure for models of size 1 to 8 genes, the optimal model size was determined using a conditional randomization test. By using logic regression, we identified candidate gene panels consisting of various subsets of the genes examined. In these analyses, sensitivity was defined as the percentage of histologically confirmed cases of CIN-3/CIS or ICC in which gene hypermethylation was detected. Specificity was defined as the percentage of histologically confirmed negative, ASCUS, or CIN-1 samples in which all genes were unmethylated.

Because we were interested in determining whether detection of hypermethylated genes could serve as the basis for cervical cancer screening, we analyzed the exfoliated cell samples rather than biopsy tissue for the presence of hypermethylated DNA. The kappa statistic was used to measure agreement between detection of hypermethylation in the exfoliated cell samples and the biopsy samples, over and above that which would be expected by chance. Kappa values of 0.00–0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80, and 0.81–1.00 are considered to represent "poor," "fair," "moderate," "substantial," and "almost perfect" agreement, respectively *(31)*.

To model the potential performance of our derived candidate "hypermethylated gene" panels in the original screening population of 2609 Senegalese women aged 35 years or older presenting to the community-based health clinics *(28)*, we extrapolated the extent of cervical disease that would be expected in the screening population with a gold standard of histologic diagnosis using the observed relationships among same-day cytology, HPV DNA detection, and histology among the 807 women from this screening population who actually underwent biopsy. This approach assumes that valid estimates will be obtained if women with similar cytology and HPV results who do and do not undergo biopsy have similar distributions of histology results. The majority of extrapolation took place in those women with normal cytology or ASCUS at screening because minimal extrapolation was needed for those women with CIN-1 or worse detected by cytology, 78% of whom had histologic evaluation of cervical disease. Statistical analyses were performed with SAS version 8.2 (SAS Institute, Cary, NC) and S-PLUS version 6 (Insightful, Seattle, WA). All statistical tests were two-sided.

Results

Demographic Characteristics of the Study Population

The goal of the present study was to assess whether detection of hypermethylation of a subset of genes of interest in exfoliated cell samples might be used to identify women with CIN-3/CIS or ICC. The mean age of the 319 women included in this study was 44.3 years (range = 35–80 years). The majority (75%) of women were premenopausal, and 70% of them did not currently practice

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contraception. All but two women reported at least one prior pregnancy, and 249 (78%) of the women reported five or more pregnancies.

Hypermethylated Genes Associated With CIN-3/CIS and ICC

DNA hypermethylation for each of the 20 genes was examined in the exfoliated cell samples. The proportion of samples in which DNA hypermethylation was detected, both overall and stratified by degree of histologic abnormality present in the matching cervical biopsy collected on the same day, is shown in Table 1. For four genes, CDH13, DAPK1, RARB, and TWIST1, the frequency of hypermethylation statistically significantly (P<.001 for each gene after Bonforroni adjustment) increased with increasing severity of neoplasia present in the cervical biopsy. Although hypermethylation was detected infrequently for six additional genes (SYK, MGMT, FHIT, ASC, CCND2, and MLH1), the hypermethylation rates were twice as high among samples from women with confirmed ICC as the rates among samples from women with CIN-1 or less. Overall, 90.1% of samples with women with ICC, 69.6% of samples with CIN-3/CIS, and 28.9% of cervical biopsies without neoplasia were noted to have hypermethylation of one or more of 10 genes of interest (CDH13, DAPK1, RARB, TWIST1, SYK, MGMT, FHIT, ASC, CCND2, and MLH1).

Because a panel that included all 10 of the above-mentioned genes had relatively low specificity (i.e., 71.1%), we next examined the sensitivity and specificity of subsets of these 10 genes for detection of CIN-3 or worse by using logic regression (Table 2). Our aim was to identify genes whose hypermethylation was predictive of ICC and of those cervical cancer precursor lesions that, if left untreated, would have a high risk of progression to ICC. Previous work has shown that, if left untreated, lesions from approximately 35%–65% of women with CIN-3/CIS, 12%–20% of women with CIN-2, and less than 5% of women with changes of CIN-1 or less would progress to ICC during a normal life span *(32)*. Therefore, genes of greatest interest were those hypermethylated in a majority of ICC and CIN-3/CIS lesions, in a lower proportion of CIN-2 lesions, and rarely detected in lesions of CIN-1 or less. Thus, genes such as DAPK1, RARB, and TWIST1 were of particular interest because they were not only more frequently hypermethylated in ICC than in normal/ASCUS biopsy samples but also were hypermethylated less often in CIN-2 and CIN-3/CIS precursor lesions than in ICC. A panel of three genes (DAPK1, RARB, and TWIST1) detected 68 (73.9%) of 92 samples of ICC and 13 (56.5%) of 23 CIN-3/CIS samples, whereas 172 (95.0%) of 181 samples with CIN-1 or less were negative for hypermethylation of all three genes (Table 2).

Leave-one-out cross-validation modeling showed that, with an estimated specificity of 95% (95% confidence interval [CI] = 94% to 95%), the three-gene panel had an estimated sensitivity of 74% (95% CI = 73% to 75%) for ICC and of 52% (95% CI = 49% to 55%) for CIN-3/CIS. The inclusion of additional genes in the three-gene model did not further improve sensitivity or specificity for detecting CIN-3/CIS or ICC (data not shown).

Detection of Hypermethylated Genes in DNA From Cervical Biopsy and Exfoliated Cell Samples

Because analysis of exfoliated cell samples collected with a cervical swab rather than that of tissue biopsy samples would have greater utility for screening purposes, we wished to assess agreement in hypermethylation of the three genes of greatest interest (DAPK1, RARB, and TWIST1) in paired samples collected on the same day. The percentage of samples in which

Table 1. Detection of hypermethylated genes in exfoliated cells collected by cervical swab according to histologic grade of cervical abnormality, among 319 women aged 35 years or older*

	Histologic grade						
Gene	Negative/ ASCUS n = 142	CIN-1 n = 39	CIN-2 n = 23	CIN-3/CIS n = 23	ICC n = 92	Overall N = 319	P_{trend}
SFN	95/104 (91.4)	31/32 (96.9)	15/15 (100.0)	17/18 (94.4)	69/70 (98.6)	227/239 (95.0)	.04
HIC1	83/129 (64.3)	25/37 (67.6)	12/19 (63.2)	14/20 (70.0)	56/79 (70.9)	190/284 (66.9)	.3
APC	47/131 (35.9)	12/37 (32.4)	9/18 (50.0)	4/20 (20.0)	28/88 (31.8)	100/294 (34.0)	.4
CDH13	25/125 (20.0)	1/28 (3.6)	1/13 (7.7)	4/19 (21.1)	41/89 (46.1)	72/274 (26.3)	<.001†
DAPK1	3/140 (2.1)	3/39 (7.7)	4/23 (17.4)	12/23 (52.2)	50/91 (55.0)	72/316 (22.8)	<.001†
CDH1	6/34 (17.7)	0/12 (0.00)	0/6 (0.00)	3/12 (25.0)	5/26 (19.2)	14/90 (15.6)	.6
RARB	4/125 (3.2)	0/29 (0.0)	0/14 (0.0)	3/19 (15.8)	34/89 (38.2)	41/276 (14.9)	<.001†
TWIST1	0/85 (0.0)	0/23 (0.0)	0/9 (0.0)	3/13 (23.1)	24/56 (42.9)	27/186 (14.5)	<.001†
SYK	5/83 (6.0)	0/17 (0.0)	0/9 (0.0)	1/16 (6.3)	11/73 (15.1)	17/198 (8.6)	.05
MGMT	3/140 (2.1)	1/39 (2.6)	0/23 (0.0)	0/23 (0.0)	10/90 (11.1)	14/315 (4.4)	.006
FHIT	5/140 (3.6)	1/39 (2.6)	1/23 (4.4)	0/23 (0.0)	7/90 (7.8)	14/315 (4.4)	.2
ASC	4/130 (3.1)	1/34 (2.9)	0/16 (0.0)	0/20 (0.0)	6/89 (6.7)	11/289 (3.8)	.3
CCND2	3/84 (3.6)	0/22 (0.0)	0/12 (0.0)	0/17 (0.0)	4/54 (7.4)	7/189 (3.7)	.4
CDKN2A	5/131 (3.8)	0/39 (0.0)	0/23 (0.0)	1/23 (4.4)	4/84 (4.8)	10/300 (3.3)	.7
CDKN2B	3/131 (2.3)	1/39 (2.6)	0/23 (0.0)	1/23 (4.4)	3/82 (3.7)	8/298 (2.7)	.5
RASSF1	3/116 (2.6)	1/28 (3.6)	0/12 (0.0)	0/19 (0.0)	1/88 (1.1)	5/263 (1.9)	.3
PRDM2	2/100 (2.0)	0/14 (0.0)	0/9 (0.0)	0/16 (0.0)	2/77 (2.6)	4/216 (1.9)	.9
VHL	1/99 (1.0)	0/34 (0.0)	1/23 (4.4)	0/21 (0.0)	1/62 (1.6)	3/239 (1.3)	.8
GSTP1	2/132 (1.5)	0/36 (0.0)	1/23 (4.4)	0/22 (0.0)	0/88 (0.0)	3/301 (1.0)	.3
MLH1	0/137 (0)	0/39 (0)	1/23 (4.4)	0/23 (0)	1/90 (1.1)	2/312 (0.6)	.3

*Number of positive samples out of total number of samples (percentage). The numbers of samples tested for each gene varies because not all genes were tested for all samples and because invalid hypermethylation tests were not included.

†Statistically significant at P<.001, the level for statistical significance determined after Bonferroni adjustment for multiple comparisons. ASCUS = atypical squamous cells of undetermined significance; CIN = cervical intraepithelial neoplasis; CIS = carcinoma in situ; ICC = invasive cervical carcinoma.

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Table 2. Estimated sensitivity and specificity and corresponding 95% confidence intervals (CIs) for a panel of hypermethylated genes to detect CIN-3/CIS or ICC*

Gene panel	No. of samples with DNA hypermethylation detected/total no. of samples (%) ICC	No. of samples with DNA hypermethylation detected/total no. of samples (%) CIN-3/CIS	No. of samples without DNA hypermethylation detected/total no. of samples (%) CIN-1 or less	Estimated sensitivity (95% CI) for ICC†	Estimated sensitivity (95% CI) for CIN-3/CIS†	Estimated specificity (95% CI) for CIN-1 or less†
DAPK1	50/92 (54.4)	12/23 (52.2)	175/181 (96.7)	54% (53% to 55%)	52% (49% to 55%)	97% (96% to 97%)
DAPK1 or RARB	64/92 (69.6)	13/23 (56.5)	172/181 (95.0)	70% (69% to 71%)	52% (49% to 55%)	95% (94% to 95%)
DAPK1 or RARB or TWIST1	68/92 (73.9)	13/23 (56.5)	172/181 (95.0)	74% (73% to 75%)	52% (49% to 55%)	95% (94% to 95%)

*CIN = cervical intraepithelial neoplasia; CIS = carcinoma in situ; ICC = invasive cervical cancer.

†Estimated sensitivities and specificities were determined by leave-one-out cross-validation procedure. 95% CIs are for binomial proportions.

concordant results were observed for exfoliated cell and biopsy samples was 88.5%, 85.0%, and 87.9% for the DAPK1, RARB, and TWIST1 genes, respectively (Table 3). The kappa statistics were 0.76, 0.64, and 0.61, respectively, indicating substantial agreement between the detection of hypermethylation in DNA from samples collected via a cervical swab and that in DNA from biopsy samples. Among discordant pairs, all three genes were more likely to be hypermethylated in tissue biopsy samples than in samples collected via a cervical swab, although the proportion of discordant pairs among the total number of pairs of samples was small (<15%).

Potential Sensitivity and Specificity of Hypermethylated Genes for the Detection of CIN-3 or Worse in a Screening Population

To examine the potential performance of the panel of hypermethylated genes in a primary screening population, we used to model the sensitivity and specificity of our derived panels consisting of one to eight genes among the 2609 participants of the screening study *(28)*. In this model, we assumed that the relationship between a specific histologic diagnosis and the frequency of hypermethylated genes observed among the 319 participants used to derive the panel of hypermethylated genes was similar to that present in the overall screening population. Likewise, we assumed that the relationship between the cytologic and histologic diagnoses seen in the 807 women who provided biopsy samples from the screening population was similar to that of the overall screening population. A single gene (DAPK1) had a sensitivity of detecting CIN-3 or worse of 53% (95% CI = 49% to 55%), with a specificity of 97% (95% CI = 96% to 97%) in our extrapolated screening population. Adding a second gene (RARB) increased the sensitivity to 58% (95% CI = 55% to 61%) but decreased the specificity to 95% (95% CI = 94% to 95%). If a three-gene panel consisting of DAPK1, RARB, and TWIST1 were to be used in a screening population to detect histologically confirmed CIN-3 or greater, we would estimate that such lesions would be detected with a sensitivity of 60% (95% CI = 57% to 63%) and a specificity of 95% (95% CI = 94% to 95%). The addition of additional genes (MGMT, SYK, ASC, MLH1, and CDH13) to the panel did not substantially improve sensitivity in our extrapolated screening population (data not shown).

Discussion

The effect of DNA hypermethylation in gene promoter regions is similar to genetic loss-of-function mutations *(33)*. In this study, we explored whether analyzing the methylation state of a panel of genes might potentially serve as the basis for a screening assay to identify women with CIN-3/CIS or ICC. We examined the methylation status of 20 genes known to be involved in regulation of oncogenesis as potential biomarkers for CIN-3/CIS or ICC. Rates of hypermethylation of 10 genes (CDH13, DAPK1, RARB, TWIST1, SYK, MGMT, FHIT, ASC, CCND2, and MLH1) were at least twice as high among samples from women with biopsy-confirmed cervical pathology as those among samples from women without such pathology. In cross-validation data sets, hypermethylation of one of three genes (DAPK1, RARB, TWIST1) had an estimated sensitivity of 74% for ICC and 52% for CIN-3/CIS, with an estimated specificity of 95%. When we modeled the performance of a panel of three (DAPK1, RARB, TWIST1) hypermethylated genes in the screening population of 2609 consecutive women who had presented to community-based health clinics, detection of hypermethylation of at least one of these three genes from exfoliated cell samples provided a sensitivity of 60% and specificity of 95% for the detection of biopsy-confirmed CIN-3/CIS or ICC. Through use of such a strategy, 7% of the screened population would have hypermethylation of one of these three genes, which is considerably lower than the percentage who tested positive for high-risk HPV types (17%) or who had ASCUS or worse on cytologic evaluation (16%). Thus, if this gene panel were used in a setting in which biopsy confirmation was deemed necessary before treatment, the number of women referred for additional testing would be less than half of that referred following other strategies. The projected sensitivity and specificity for identification of women with CIN-3 or worse lesions using our three-gene panel compare favorably with the reported sensitivity and specificity of cytology and detection of HPV for identification of

Table 3. Detection of hypermethylation of three genes in paired exfoliated cells collected via cervical swabs and tissue biopsy samples

Gene	Hypermethylation result* (exfoliated cells/tissue biopsy) Negative/negative	Negative/positive	Positive/negative	Positive/positive	Kappa statistic (95% CI)
DAPK1	74	11	4	42	0.76 (0.64 to 0.87)
RARB	75	11	6	21	0.61 (0.44 to 0.78)
TWIST1	48	5	3	10	0.64 (0.41 to 0.87)

*A negative result was obtained if the methylated control DNA, but not the test sample DNA or the unmethylated control DNA, was amplified during the polymerase chain reaction (PCR). A positive result was obtained if the methylated control DNA and the test sample DNA, but not the unmethylated control DNA, were amplified during the PCR.

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such lesions. Kulasingam et al. *(6)* found that a diagnosis of AS-CUS or worse on a thin-layer Pap test in a population of women aged 30 years or older identified women with lesions graded CIN-3 or worse with a sensitivity of 50% and a specificity of 86%. In the same study, a PCR-based assay for high-risk HPV types identified women with CIN-3 or worse with a sensitivity of 80% and a specificity of only 87%. Thus, if this gene panel was used in a setting in which biopsy confirmation were deemed necessary before treatment, we would expect that, compared with other strategies, fewer women would be referred for additional testing, with similar numbers of women with CIN-3 or worse identified.

Our study is the first, to our knowledge, to examine a panel of genes in which hypermethylation was detected in a large number of exfoliated cell samples and in matched biopsy samples collected the same day. Previous studies have reported the presence of promoter hypermethylation in cervical cancer cell lines *(34)* and in a series of U.S. women with and without cervical neoplasia *(26,27,35,36)*. Chen et al. *(34)* detected hypermethylation of E-cadherin (CDH1) in all five cell lines examined and 8 of 20 tumor samples. Hypermethylation of RASSF1A (RASSF1) was detected in 11 of 33 squamous cell cancers and none of 11 normal control samples *(36)*. Tripathi et al. *(37)* detected hypermethylation of p16 (CDKN2A) in 3 of 46 cervical lesions. In a larger study examining a greater number of genes, Dong et al. *(26)* reported that 70% of 53 ICC case subjects but none of 24 normal hysterectomy specimens had hypermethylation of the CDKN2A, DAPK1, HIC1, APC, CDH1, or MGMT gene. Virmani et al. *(27)* found that the CDKN2A, RARB, FHIT, GSTP1, MGMT, or MLH1 gene was hypermethylated in 74% of 19 ICC samples, 71% of 17 CIN-2 or CIN-3 samples, and 30% of 37 samples of CIN-1 or less. Narayan et al. *(35)* examined 16 genes (CDH1, DAPK1, RARB, HIC1, FHIT, RASSF1, APC, CDKN2A, MGMT, BRCA1, TP73, TIMP3, GSTP1, MLH1, CDKN2B, and RB1) in 82 primary cervical carcinomas and eight normal control samples and detected hypermethylation of at least 1 of 14 of those genes (CDKN2B and RB1 were not methylated) in 87% of the ICC samples. It is interesting that these previous studies among non-African women reported finding hypermethylation of many of the same genes identified in our study among African women (e.g., DAPK1 and RARB) as associated with the presence of CIN-3 or worse, suggesting that our findings may be generalizable to most populations.

Although the frequency of hypermethylation of many of the genes examined in our study was similar to that reported by others *(26,27,35)*, we observed some differences. We detected hypermethylation of MGMT, FHIT, GSTP1, and MLH1 in ICC less frequently than it was detected by Virmani et al. *(27)* but at a frequency similar to that reported by Dong et al. *(26)* and Narayan et al. *(35)*. Because most studies used similar hypermethylation detection assays, observed differences in hypermethylation frequencies likely reflect other differences, such as differences in sample processing, e.g., the use of formalin-fixed paraffin-embedded tissues rather than of frozen tissues or the use of exfoliated cell samples preserved in either ethanol-based or other fixatives rather than those preserved in STM media. Furthermore, differences in the age of study subjects, racial and ethnic background, cancer stage, histologic type or degree of differentiation, previous medical or surgical treatment, or previous exposures relevant to the development of malignancy might also be related to the differing frequencies of hypermethylation.

Because information regarding these factors has not been presented in the present study or in previous studies, it may not be possible to directly compare our findings with those of others, and thus our findings may not be generalizable to other populations. Further studies in other populations are needed to confirm and extend our findings. In our study, participants were multigravid West African women aged 35 years or older, with little history of birth control, alcohol, or tobacco use and no previous cervical screening. Women with cancer generally presented with large tumors and late-stage disease. Because hypermethylation of any one gene generally occurred infrequently, examination of large sample sizes will be needed to accurately characterize the association between promoter hypermethylation of particular genes and degree of neoplasia. Furthermore, little is known concerning risk factors for the presence of hypermethylation of genes associated with cancer in the absence of neoplasia.

Our findings support the notion that a panel of hypermethylated genes could serve as the basis of a relatively sensitive and specific primary screening assay to detect cervical cancer and its precursor lesions. However, our study has several potential limitations. First, we were not able to incorporate into the model information concerning the relative level of hypermethylation of each specific gene. It is possible that other gene combinations, which may have increased sensitivity and specificity, will be identified through the use of real-time PCR-based assays such as MethyLight *(38)*, which provide information on the relative level of hypermethylation of each specific gene examined. Second, we limited our search for useful hypermethylated genes to an assessment of 20 genes that had been previously reported to be associated with either cervical cancer or cancers at other sites. Identification of additional novel CpG islands that are specifically associated with cervical cancer will be needed to construct a panel with higher sensitivity that maintains high specificity, and studies examining detection of such a panel of genes using recently developed quantitative assays should be undertaken. In addition, assays that are based on the identification of changes in the function of genes central to the maintenance of genetic stability will offer the possibility of identifying the subset of precursor lesions that carry a high risk of progression to ICC. Perhaps most important, longitudinal studies will be needed to determine whether women without CIN-3 or ICC who nonetheless test positive for the presence of specific hypermethylated genes are truly at increased risk of progression to higher-grade lesions. To evaluate the performance of these genetic markers, findings from our study and future studies should be confirmed in other populations and risk groups of women.

Detection of gene hypermethylation is currently a research assay. However, if detection of hypermethylation of these or other genes proves to be highly predictive of CIS-3 or ICC, we anticipate that an inexpensive clinical assay will be developed for use worldwide. A similar situation existed 15 years ago for detection of HPV DNA, which then could be detected only by Southern transfer hybridization (an assay that is similar in cost and difficulty to the assays now being used for detection of hypermethylation). After it was shown that detection of high-risk types of HPV DNA had clinical utility for cervical cancer screening, a clinical assay was produced and is now used widely. An inexpensive assay for detection of HPV DNA is now being developed for use in resource-poor settings. We anticipate a similar scenario will occur with detection of hypermethylated genes that are predictive of CIS-3 or ICC.

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Appendix. Gene symbol, name, function, primer sequences, and annealing temperature (Ta) for the methylation-specific polymerase chain reaction (PCR) analyses

Gene symbol	Gene name	Gene function	Primer sequence*	Ta†	Reference
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, inhibits CDK4) p16,	Cell cycle control	(U) TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	60	(*39*)
			(M) TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA	60	(*39*)
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Cell cycle control	(U) TGTGATGTGTTTGTATTTTGTGGTT CCATACAATAACCAAACAACCAA	60	(*39*)
			(M) GCGTTCGTATTTTGCGGTT CGTACAATAACCGAACGACCGA	60	(*39*)
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Adhesion	(U) TAATTTTAGGTTAGAGGGTTATTGT CACAACCAATCAACAACACA	52	(*39*)
			(M) TTAGGTTAGAGGGTTATCGCGT TAACTAAAAATTCACCTACCGAC	52	(*39*)
VHL	Von Hippel–Lindau syndrome	Cell cycle control, apoptosis	(U) GTTGGAGGATTTTTTTGTGTATGT CCCAAACCAAACACCACAAA	60	(*39*)
			(M) TGGAGGATTTTTTTGCGTACGC GAACCGAACGCCGCGAA	60	(*39*)
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*)	DNA repair	(U) TTTTGATGTAGATGTTTTATTAGGGTTGT ACCACCTCATCATAACTACCCACA	60	(*40*)
			(M) ACGTAGACGTTTTATTAGGGTCGC CCTCATCGTAACTACCCGCG	60	(*40*)
DAPK1	Death-associated protein kinase 1	Apoptosis	(U) GGAGGATAGTTGGATTGAGTTAATGTT CAAATCCCTCCCAAACACCAA	60	(*41*)
			(M) GGATAGTCGGATCGAGTTAACGTC CCCTCCCAAACGCCGA	60	(*41*)
MGMT	O-6-methylguanine-DNA methyltransferase	DNA repair	(U) TTTGTGTTTTGATGTTTGTAGGTTTTTGT AACTCCACACTCTTCCAAAAACAAAACA	60	(*41*)
			(M) TTCGACGTTCGTAGGTTTTCGC GCACTCTTCCGAAAACGAAACG	60	(*41*)
FHIT	Fragile histidine triad gene	Cell cycle control	(U) TTGGGGTGTGGGTTTGGGTTTTTATG CATAAACAACACCAACCCCACTA	60	(*42*)
			(M) TTGGGGCGCGGGTTTGGGTTTTTACGC CGTAAACGACGCCGACCCCACTA	60	(*42*)
GSTP1	Glutathione S-transferase pi	Cellular detoxification	(U) GATGTTTGGGGTGTAGTGGTTGTT CCACCCCAATACTAAATCACAACA	55	(*43*)
			(M) TTCGGGGTGTAGCGCTCGTC GCCCCAATACTAAATCACGACG	55	(*43*)
HIC1	Hypermethylated in cancer 1	Cell cycle control	(U) AACGTCCATAAACAACAACGCG CACCCTAACACCACCCTAAC	60	(*26*)
			(M) TCGGTTTTCGCGTTTTGTTCGT GCGATACCCGCCCTAACGCCG	60	(*26*)
APC	Adenomatosis polyposis coli	Cell cycle control, adhesion	(U) AATTTGTTGGATGTGGATTAGGGT AACCACATATCAATCACATACA	60	(*26*)
			(M) CGTTGGATGCGGATTAGGGC CCACATATCGATCACGTACG	60	(*26*)

(*Table continues*)

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Appendix (continued).

Gene symbol	Gene name	Gene function	Primer sequence*	Ta†	Reference
RARB	Retinoic acid receptor, beta	Cell differentiation	(U) TTGAGAATGTGAGTGATTTA AACCAATCCAACCAAAACAA	60	*(44)*
			(M) TCGAGAACGCGAGCGATTCG GACCAATCCAACCGAAACGA	60	*(44)*
SFN	Stratifin	Cell cycle control, cell proliferation	(U) ATGGTAGTTTTTATGAAAGGTGTT CCCTCTAACCACCCACCACA	55	*(45)*
			(M) TTCGGGGTGTAGCGCTCGTC CCTCTAACCGCCCACCACG	55	*(45)*
CCND2	Cyclin D2	Cell cycle control	(U) GTTATGTTATGTTTGTTGTATG TAAAATCCACCAACACAATCA	55	*(21)*
			(M) TACGTGTTAGGGTCGATCG CGAAATATCTACGCTAAACG	55	*(21)*
RASSF1	Ras association (RalGDS/AF-6) domain family 1	Cell cycle control	(U) GGTTTTGTGAGAGTGTGTTTAG CACTAACAAACACAAACCAAAC	55	*(46)*
			(M) GGGTTTTGCGAGAGCGCG GCTAACAAACGCGAACCG	55	*(46)*
ASC	Apoptosis-associated speck-like protein containing a CARD	Apoptosis	(U) GGTTGTAGTGGGGTGAGTGGT CAAAACATCCATAAACAACAACACA	55	*(47)*
			(M) TTGTAGCGGGGTGAGCGGC AACGTCCATAAACAACAACGCG	55	*(47)*
CDH13	Cadherin 13, H-cadherin (heart)	Adhesion	(U) TTGTGGGGTTGTTTTTTGT AACTTTTCATTCATACACACA	55	*(48)*
			(M) TCGCGGGGTTCGTTTTTCGC GACGTTTTCATTCATACACGCG	55	*(48)*
SYK	Spleen tyrosine kinase	Cell proliferation	(U) ATTTTGTGGGTTTTGTTTGGTG ACTTCCTTAACACACCCAAAC	55	*(49)*
			(M) CGATTTCGCGGGTTTCGTTC AAAACGAACGCAACGCGAAAC	55	*(49)*
PRDM2	PR domain containing 2, with ZNF domain	Transcription regulation	(U) TGGTGGTTATTGGGTGATGGT ACTATTTCACCAACCCCAAGA	68	*(50)*
			(M) GTGGTGGTTATTGGGCGACGGC GCTATTTCGCCGACCCCGACG	68	*(50)*
TWIST	Twist homolog 1 (acrocephalosyndactyly 3; Saethre–Chotzen syndrome) (*Drosophila*)	Cell differentiation	(U) TTTGGATGGGGTTGTTATTGT CCTAACCCAAACAACCAACC	55	*(51)*
			(M) TTTCGGATGGGGTTGTTATC AAACGACCTAACCCGAACG	55	*(51)*

*Primer sequences were specifically designed to detect unmethylated (U) or methylated (M) genes.
†Annealing temperature for methylation-specific PCR.

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Notes

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