DNA Methylation in Anal Intraepithelial Lesions and Anal Squamous Cell Carcinoma
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Abstract Purpose: Anal intraepithelial neoplasia is associated with human papillomavirus infection and may progress to invasive squamous cell carcinoma (SCC), which is increasing in immunocompromised patients. We hypothesize that anal intraepithelial neoplasia is associated with abnormal DNA methylation and that detection of these events may be used to improve screening programs.

Experimental Design: Seventy-six patients were identified who underwent anal cytology screening and subsequent biopsy at our institution between 1999 and 2004. The specimens from these patients included 184 anal biopsies [normal, n = 57; low-grade squamous intraepithelial lesion (LSIL), n = 74; high-grade squamous intraepithelial lesion (HSIL), n = 41; and invasive SCC, n = 12] and 37 residual liquid-based anal cytology specimens (normal, n = 11; LSIL, n = 12; HSIL, n = 14). The methylation status of the following genes was determined for each biopsy and cytology sample using real-time methylation-specific PCR: HIC1, RASSF1, RARB, CDKN2A, p14, TP73, APC, MLH1, MGMT, DAPK1, and IGSF4.

Results: Methylation-specific PCR analysis of biopsy samples revealed that DNA methylation was more common in SCC and HSIL than LSIL and normal mucosa. Specifically, methylation of IGSF4 and DAPK1 was prevalent in SCC (75% and 75% of cases, respectively) and HSIL (59% and 71%, respectively) but was absent in LSIL and normal biopsy samples. Methylation profiles of cytologic samples were similar to those found in the biopsy samples.

Conclusions: Aberrant DNA methylation is a frequent event in anal HSIL and SCC. Methylation of IGSF4 and DAPK1 is specific for HSIL and SCC, and may serve as a useful molecular biomarker.

It has been estimated that there will be 3,990 new cases of invasive anal carcinoma in the U.S. in the year 2005 (1). Although anal squamous cell carcinoma (SCC) is relatively rare, it is the fourth most common reported malignancy among men with HIV infection (2). In these individuals, there is an association between anal cancer precursor lesions and low CD4+ counts (3–5). It has recently been shown that anal cancer precursor lesions are highly prevalent in HIV-positive men who have sex with men, despite immune restoration by highly active antiretroviral therapy (6).

The pathogenesis of anal cancer is poorly understood, but shares features with cervical carcinoma, including an association with human papillomavirus (HPV) infection (7). Like cervical cancer, anal cancer is preceded by HPV-associated precursor lesions termed anal intraepithelial neoplasia (AIN). These precursor lesions can be divided into three groups, based on the degree of cytologic and histologic atypia, designated AIN I, II, and III (8). AIN I is also termed low-grade squamous intraepithelial lesion (LSIL), whereas AIN II and AIN III are often combined as high-grade squamous intraepithelial lesions (HSIL). These categories are important because, in the cervix, and possibly the anus, cancers are believed to be preceded by HSIL, whereas LSIL is not thought to progress directly to cancer and may spontaneously regress.

Screening protocols for anal cancers have been proposed, but unfortunately, these have not been widely implemented. These protocols rely on microscopic analysis of a sample of anal cells obtained by rotating a swab in the anal canal. Patients with abnormal anal cytology are typically referred for biopsy of suspicious lesions. Detection rates of anal cytology for AIN vary from study to study, typically ranging from 34% to 81% (9). Although a cytologic diagnosis of HSIL is highly specific for biopsy-proven HSIL, sensitivity is low. Use of a lower cytologic cutoff than HSIL is more sensitive for HSIL detection but lacks specificity. Due to this lack of specificity, the current screening protocols rely on costly, invasive biopsies to identify high-risk lesions. Molecular biomarkers of HSIL that would maintain screening sensitivity but reduce the rate of unnecessary biopsies would be valuable. Unfortunately, very little is known about the molecular or cellular biology of AIN (10).
DNA methylation, which involves the addition of a methyl group to cytosine nucleotides, has an important role in tumorigenesis (11, 12). The promoter regions of many tumor suppressor genes are specifically methylated in tumors but not in normal tissues, resulting in repression of tumor suppressor gene expression. Methylation of tumor suppressor genes has been detected in virtually every type of malignancy, including carcinomas, sarcomas, lymphomas, and gliomas, and the profile of methylated genes seems relatively specific for each tumor type (13).

To date, there have been no published reports of DNA methylation in anal neoplasia; however, there have been several studies of DNA methylation in cervical cancer, another HPV-related neoplasm. Further studies are necessary to elucidate the biological role of DNA methylation in anal neoplasia and to test its utility as a biomarker. In this study, we examined the methylation status of a panel of candidate genes in a spectrum of anal intraepithelial lesions and invasive anal carcinomas from biopsy samples and anal cytology samples.

Materials and Methods

Patient population. Between January 1, 1999 and July 1, 2004, the Johns Hopkins Cytopathology Laboratory received 470 anal cytology samples from clinicians involved in anal neoplasia screening of high-risk patients. Of these patients, 144 subsequently received high-resolution anoscopy and biopsies, most of which were done by Dr. Ciro Martins (coauthor). After obtaining Institutional Review Board approval, we identified 76 patients from this cohort who had sufficient paraffin-embedded material in the Johns Hopkins Pathology Department archive for methylation-specific PCR analysis. This population was predominantly HIV-positive (95%), male (96%), with homosexual sex as a risk behavior for HIV infection (95%), and a mean age of 43 years. The racial/ethnic distribution was 40% African-American, 51% Caucasian, 4% Hispanic, and 5% other. The specimens included 172 anal biopsies from these 76 patients [normal, n = 57; AIN I (LSIL), n = 74; AIN II-III (HSIL), n = 41]. In addition, we identified 12 patients with invasive anal SCC who had specimens in the surgical pathology archive. We also identified 37 patients from the cytopathology archives who had residual liquid-based anal cytology specimens available for molecular analysis (normal, n = 11; LSIL, n = 12; HSIL, n = 14). Eleven of these patients also had biopsy specimens that were included in our analysis.

Anal cytology sampling and analysis. Exfoliated cells were collected by clinicians as part of routine care by inserting a saline-moistened Dacron swab –2 in. into the anal canal and slowly removing it in a circular motion. The swab was thoroughly rinsed in 10 mL of SurePathPreservative Fluid (TriPath Imaging, Inc., Burlington, NC) and delivered to the Johns Hopkins Cytology Laboratory for processing. For routine cytologic analysis, a single glass slide was prepared from 2 mL of the 10 mL SurePathPreservative Fluid sample using the SurePath technology (TriPath Imaging), then stained with the Papanicolaou stain and screened for cytologic atypia by a cytopathologist. The specimens were reviewed by a board-certified cytopathologist (D.P. Clark) and reported as normal, LSIL, or HSIL using criteria outlined in the Bethesda System 2001 (14). After 2 cc was removed in the clinical cytopathology laboratory for routine processing, the remaining 8 cc, which is typically discarded, was used for methylation analysis.

High-resolution anoscopy. Anoscopic examination was done on all patients by one of the authors (C.R. Martins). Briefly, a clear plastic disposable anoscope was inserted through the anal canal into the rectum with the aid of a water-soluble lubricant. A swab that had been wrapped in gauze and dipped in 3% acetic acid was then placed inside the anoscope, which was subsequently removed, leaving the acetic acid–soaked swab inside the anal canal. After 1 minute, the swab was removed, and the anoscope completely reinserted. After the transformation zone was completely examined with a colposcope (CervellanceScope, CooperSurgical, Inc., Trumbull, CT), suspicious lesions were biopsied using endoscopic forceps, fixed in buffered formalin and delivered to the Johns Hopkins Surgical Pathology division for analysis (15).

Histologic examination. All anal biopsies were examined by the staff surgical pathologists at the Johns Hopkins Hospital as part of routine clinical care. H&E-stained slides were cut from formalin-fixed paraffin-embedded biopsy specimens and reported as normal, AIN I (LSIL), or AIN II-III (HSIL) using accepted criteria, which are based on cervical neoplasia grading systems (8, 16). To minimize interobserver bias in the histologic interpretation of AIN (17), all biopsies were blindly reviewed by a second anatomic pathologist (J. Zhang). Any cases with a discrepancy between these two interpretations were adjudicated blindly by a third anatomic pathologist (D.P. Clark). Final arbitrated diagnoses were used as the final pathologic classification in all analyses.

Nucleic acid extraction from clinical samples. After routine clinical analysis of the anal biopsies was complete, the paraffin-embedded tissue was retrieved from the pathology archive and five 10-μm sections were cut for nucleic acid extraction. Five-micrometer sections flanking the material used for DNA extraction were also cut from the block, stained with H&E, and examined by a pathologist (J. Zhang) to confirm that the diagnosis remained the same as the original diagnosis in the tissue used for molecular analysis. Paraffin was removed with xylene and DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA) and quantitated by spectrophotometric analysis. For the cytologic samples, each 8 cc sample was centrifuged and washed twice in PBS to remove the SurePathPreservative Fluid. DNA was extracted from the sample using the DNeasy kit (Qiagen) and quantitated by spectrophotometric analysis.

Methylation profiling of anal biopsy samples. Real-time methylation-specific PCR was used to determine the methylation status of each of the following genes: DAPK1, IGF5 (TSLC1), MLH1, HIC1, RARB, p14, TP73, MGMT, RASSF1, APC, CDKN2A (p16). DNA (1 μg) from each biopsy was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) in preparation for methylation-specific PCR. The methylation-specific PCR reactions used 10 ng of the sodium bisulfite–treated DNA in 12.5 μL of 2× Brilliant SYBR Green QPCR Master Mix (dNTPs, Taq DNA polymerase, 2.5 mmol/L MgCl2; Stratagene, La Jolla, CA), 30 nmol/L ROX reference dye, and 300 nmol/L of forward and reverse primers, in a final volume of 25 μL. Real-time methylation-specific PCR was done on a Mx3000P Thermocycler (Stratagene). The primer sequences used were described in the referenced publications as the methylated sense and antisense primers, and were previously validated (18–22). The real-time methylation-specific PCR reaction used the following conditions: segment one—one cycle at 95°C for 10 minutes; segment two—65 cycles at 95°C for 30 seconds, then 60°C to 64°C (depending on primers) for 30 seconds, then 72°C for 30 seconds; segment three—final extension at 72°C for 30 seconds. After the final extension step, a dissociation curve was analyzed that consisted of one cycle at 95°C for 1 minute, then 55°C for 30 seconds, then in an increase in temperature to 95°C with data collection throughout. Normal male WBC genomic DNA (Novagen, Madison, WI) was used as a negative DNA control and CpGenome Universal Methylated DNA, Human Male (Chemicon International, Temecula, CA) was used as a positive control. Primers that recognize both methylated and unmethylated templates from a region in the MYOD1 gene that lacks CpG islands were used to confirm the presence of PCR-amplifiable DNA in each sample. Each gene was scored as positive or negative for methylation. The individual target gene was considered positive for methylation if there was a PCR product detected at the appropriate temperature of the dissociation curve, and the gene was considered negative if there was no peak at the appropriate temperature of the dissociation curve. Samples were considered noninformative if there was no PCR product generated from the
MYOD1 control. Before beginning our research with anal samples, studies were conducted to assess the sensitivity of our real-time methylation-specific PCR assay using varying proportions of methylated and unmethylated control DNA. Serial dilutions of methylated control DNA ranging from 10 ng (−3,000 cell equivalents of DNA) to 0.001 ng (<1 cell equivalent of DNA) were mixed with an excess of unmethylated control DNA (to a total of 10 ng) in methylation-specific PCR reactions for each gene. The minimal amount of DNA detected by methylation-specific PCR ranged from 0.001 to 0.1 ng of DNA for all genes except RARB, which detected <1 ng.

Statistical analysis. We used the stratified Cochran-Mantel-Haenszel test, which is sensitive to associations in two-way classifications of ordered categorical outcomes, to assess significance of the association between pathologic diagnosis (HSIL/LSIL/negative) and methylation status (methylated/not methylated) for each gene and for the total number of methylated genes. We also calculated the mean number of genes methylated for each diagnosis category, and compared the distributions of the number of genes methylated across diagnostic categories by a generalized estimating equations score test.

We used receiver-operating characteristic (ROC) analysis, with area under the ROC curve as a measure of test performance, to identify a subset of genes whose positive methylation status could distinguish HSIL from LSIL and negative samples (23). In analyzing the data, the methylation status of the tumor suppressor gene(s) in HSIL samples was compared with the methylation status of the tumor suppressor gene(s) in LSIL and negative samples combined. Based on the distribution of the gene(s) methylated for HSIL samples versus combined LSIL/negative samples, the sensitivity and specificity was calculated for each cutoff value and ROC curves were plotted as sensitivity versus 1 − specificity. The area under the ROC curve is a measure of test performance aggregated over all cutoff values. Possible areas range from 100% (a perfect test) through 50% (a test with no discriminating ability) to 0 (test is perfect if given the opposite interpretation). The area under the ROC curve was estimated for each gene separately (11 analyses, one for each gene) and for the sum of all tumor suppressor gene methylation indices, and for the sums of all pairs, triplets, and quadruplets of gene methylation indices.

Because the data represent 172 biopsy samples from 76 subjects, the individual samples are not statistically independent, and the conventional SE estimate of the nonparametric ROC area is not valid (24). To obtain a correct estimate, we used a bootstrap resampling method (25). After first computing the ROC area from the 172 samples, we selected 200 samples of size 76, with replacement, from the 76 independent subjects, and computed the ROC area from each resampled data set. These resamples would consist of varying numbers of biopsies, depending on which subjects were randomly chosen to be in them. We then computed the standard deviation of the ROC estimates from the 200 bootstrap samples and used this standard deviation as the standard error in computing P values by a normal z test. We applied this process first for each gene separately, for the sum of methylation indicators across all genes, for the sum of each pair of genes, for the sum of each triplet of genes, and for the sum of each quadruplet of genes. Once we determined the best individual gene, two-gene combination, three-gene combination, and four-gene combination, based on the largest area under the ROC curve, we tested whether four genes improved over three genes, three genes improved over two, and two genes improved over one gene, using the nonparametric ROC estimate with bootstrapped standard errors. We did the Cochran-Mantel-Haenszel and generalized estimating equations score tests in SAS Version 8 (SAS Institute, Cary, NC), and the ROC analyses in S-Plus Version 6.0 (Insightful Corporation, Seattle, WA).

Results

Aberrant host cell DNA methylation in AIN and invasive squamous carcinoma. We hypothesized that AIN contains abnormally methylated genes, and that these aberrant DNA methylation events are more frequent in HSIL than LSIL or normal anal mucosa. In order to test this hypothesis, we used real-time methylation-specific PCR to analyze the methylation status of 11 candidate genes (DAPK1, IGSF4, MLH1, HIC1, RARB, p14, TP73, MGMT, RASSF1, APC, and CDKN2A) in a set of 172 anal biopsies from a cohort of 76 patients who underwent high-resolution anoscopy at our institution. These candidate genes were selected because of their high prevalence of methylation in other carcinomas, particularly cervical carcinoma, as well as the availability of validated methylation-specific PCR primers. There was no significant difference in the mean age of patients from the analyzed groups (normal, 45.6 years; LSIL, 42.5 years; HSIL, 44.2 years; and SCC, 46.6 years; all P values >0.05). These 172 specimens (normal, n = 57; LSIL, n = 74; HSIL, n = 41) each contained from 0 to 7 methylated genes from the 11-gene panel, with generally more genes methylated as the grade of the lesion increased from normal mucosa to LSIL and HSIL (Table 1). The Cochran-Mantel-Haenszel test for correlation of diagnosis and number of methylated genes suggested a strong association between methylation status and diagnosis (P = 0.0003). The mean number of methylated genes in the specimens with either LSIL or no disease was 1.13, and the mean number in the HSIL specimens was 3.17 (generalized estimating equations score test, P = 0.0001). In Table 1, we have also shown the prevalence of individual gene methylation in normal anal mucosa, LSIL, and HSIL samples. The genes most commonly methylated in HSIL included DAPK1 (71% prevalence), IGSF4 (59%), and RARB (46%). In this set of samples, DAPK1 and IGSF4 methylation was entirely absent in normal anal mucosa and LSIL. RARB methylation was absent in normal anal mucosa and present in only 12% of LSIL biopsies. The Cochran-Mantel-Haenszel test for association of methylation status with pathologic diagnosis was significant for these three genes (P < 0.001) as well as for MGMT (P = 0.01), and methylation status was positively associated with severity of the lesion. In order to determine if the DNA methylation profiles of invasive anal
was positively associated with methylation status and the area each gene separately. For all but combination (Table 2). We first computed ROC curves for measure of test performance for each gene separately and in we used ROC analysis and area under the ROC curve as a distinguished HSIL from LSIL and normal biopsy samples, mine the combination of methylated genes that best

dna methylation in anal cytologic samples. To be useful as a screening test, these methylation markers must be compatible with a less invasively obtained sample than a biopsy, such as the exfoliated cell sample collected for anal cytopathology. However, this sample contains a more heterogeneous cell population than a biopsy sample directed to visible lesions. Therefore, in order to determine if aberrant DNA methylation events can be detected in anal cytology specimens, we did methylation-specific PCR on the previously described panel of genes in 37 liquid-based, biopsy-confirmed, anal cytology samples, including cytologically normal (n = 11), LSIL (n = 12), and HSIL (n = 14) samples. The results from the cytologic samples are shown in Table 3. As in the biopsy samples, the most commonly methylated genes in HSIL included DAPK1 (71%), IGSF4 (64%), and RARB (64%). Although the sample size is small, these data indicate that: (a) anal cytology samples in SurePath Preservative Fluid are suitable for methylation-specific PCR; (b) DNA methylation is not commonly found in normal cytologic samples; (c) DNA methylation can be detected in cytologic samples containing LSIL and HSIL cells; (d) methylation profiles from AIN in cytologic samples are similar to those found in biopsy samples.

Correlation of methylation data from cytology and biopsy specimens. In 11 of the previously described patients, we obtained methylation data on both a cytology sample and the subsequent biopsy samples, giving us the opportunity to

Table 2. ROC analysis of methylation markers to distinguish anal HSIL from combined LSIL/negative samples

<table>
<thead>
<tr>
<th>Tumor suppressor gene</th>
<th>Area under ROC curve*</th>
<th>P †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPK1</td>
<td>85% (4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IGSF4</td>
<td>77% (4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RARB</td>
<td>70% (4%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MGMT</td>
<td>58% (4%)</td>
<td>0.035</td>
</tr>
<tr>
<td>p14</td>
<td>55% (3%)</td>
<td>0.048</td>
</tr>
<tr>
<td>MLH1</td>
<td>51% (2%)</td>
<td>0.66</td>
</tr>
<tr>
<td>HIC1</td>
<td>54% (4%)</td>
<td>0.32</td>
</tr>
<tr>
<td>TP73</td>
<td>52% (2%)</td>
<td>0.31</td>
</tr>
<tr>
<td>RASSF1</td>
<td>56% (4%)</td>
<td>0.20</td>
</tr>
<tr>
<td>APC</td>
<td>47% (3%)</td>
<td>0.30</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>46% (5%)</td>
<td>0.46</td>
</tr>
<tr>
<td>Two-genecombination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPK1/IGSF4</td>
<td>96% (2%) †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Three-genecombination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPK1/IGSF4/RARB</td>
<td>96% (2%) †</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Four-genecombination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPK1/IGSF4/RARB/p14</td>
<td>96% (2%) † ‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All genes (N = 11)</td>
<td>86% (3%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Values are area under the ROC curve with SE in parenthesis.
† P value represents a statistically significant difference from 50%.
‡ P = 0.005, DAPK1/IGSF4 versus DAPK1.
§ P = 0.88, DAPK1/IGSF4/RARB versus DAPK1/IGSF4.
∥ P = 0.15, DAPK1/IGSF4/RARB/p14 versus DAPK1/IGSF4/RARB.

Table 3. Prevalence of DNA methylation in anal cytology samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Total number of samples</th>
<th>Number of samples positive for methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAPK1</td>
<td>IGSF4</td>
</tr>
<tr>
<td>Normal</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>LSIL</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>HSIL</td>
<td>14</td>
<td>10 (71)</td>
</tr>
</tbody>
</table>

squamous carcinoma were similar to the profiles of AIN, we did methylation-specific PCR on tumor samples from 12 patients. The average number of methylated genes was 3.83 for the SCC samples, which was not significantly different than the average number of methylated genes in the HSIL biopsies (P = 0.53). As in AIN, the genes that were most prevalently methylated included DAPK1 (75% prevalence), IGSF4 (75%), and RARB (42%); Table 1).

Receiver-operating characteristic analysis. In order to determine the combination of methylated genes that best distinguished HSIL from LSIL and normal biopsy samples, we used ROC analysis and area under the ROC curve as a measure of test performance for each gene separately and in combination (Table 2). We first computed ROC curves for each gene separately. For all but APC and CDKN2A, diagnosis was positively associated with methylation status and the area exceeded 50%. Five of the 11 genes gave ROC areas significantly different from 50%: DAPK (area = 85%; SE = 4%), IGSF4 (area = 77%; SE = 4%), RARB (area = 70%; SE = 4%), MGMT (area = 58%; SE = 4%) and p14 (area = 55%; SE = 3%). The ROC area for the sum of all 11 genes was 86% with SE = 3%. The best pair was DAPK/IGSF4 (area = 96%; SE = 2%). Among triplets of genes, the best was DAPK/IGSF4/RARB (area = 96%; SE = 2%), and among quadruplets, the best was DAPK/IGSF4/RARB/p14 (area = 96%; SE = 2%). The nominal P value for comparing the best pair to the best single gene was P = 0.005, suggesting that adding a second gene improved test performance. Adding a third gene to the best pair (P = 0.88) or a fourth to the best triplet (P = 0.15) did not significantly increase the area under the ROC curve. We conclude that although the methylation status of several genes correlates with biopsy diagnosis, the best possible pair is DAPK and IGSF4 together, and the addition of further genes does not improve on the value of this combination in these biopsy samples. We repeated this analysis for the comparison of 12 SCC specimens to the 28 HSIL specimens. Because of the similarity of their methylation profiles, no single-gene ROC area was significantly different from 50%, and the ROC area from the best pair of genes was not significantly larger than the area from the best single gene.
compare the methylation profiles of these two types of samples. In five of these cases there was concordance between the cytologic diagnosis and the biopsies (three LSIL and two HSIL); there was discordance between these diagnoses in six cases (one LSIL cytology/HSIL biopsy; five HSIL cytology/LSIL or normal biopsy; Table 4). We compared the individual gene methylation status from the cytology sample with the individual gene methylation status from all the biopsies in each patient (i.e., the histology was considered positive for methylation if any of the patient’s biopsies were positive). Overall, there was 63% concordance between the methylation results from the cytology samples and the biopsies (11% cytology- and histology-positive; 89% cytology- and histology-negative; data not shown). Thirty-seven percent of the cytology and histology cases were discordant in their methylation status (71% cytology-positive and histology-negative; 29% cytology-negative and histology-positive). If we limited our analysis to the methylation status of DAPK1 and IGSF4, we found concordance between the cytology and histology methylation profiles in 6 of the 11 patients (Table 4). Among the discordant samples, the cytology sample was positive for DAPK1 or IGSF4 methylation and the biopsy negative for DAPK1 or IGSF4 methylation in three of these cases. This suggests that the cytology sample contains aberrantly methylated DNA that was not present in the biopsy. Interestingly, in each of these methylation-discordant cases, the cytologic diagnosis was HSIL, whereas the biopsy diagnoses were LSIL or normal, also suggesting that the HSIL was not sampled in the biopsy.

**Discussion**

DNA methylation is increasingly recognized as an important cellular event during tumorigenesis. Until our current study, the role of DNA methylation in AIN and anal SCC was unknown. We have found that normal anal mucosa has a very low prevalence of DNA methylation in the 11-gene panel we analyzed, whereas HSIL and SCC have a higher prevalence of DNA methylation. LSILs have a prevalence of DNA methylation that is intermediate between normal mucosa and HSIL. In particular, we have found that DAPK1, IGSF4, and RARB methylation are significantly associated with HSIL and SCC, relative to LSIL and normal mucosa.

Our data add molecular validation to the division of AIN into two categories, LSIL and HSIL, which have been divided based predominantly on their histologic features. The similarity between the methylation profiles of HSIL and SCC argue that HSIL is indeed a precursor of SCC. Although our data suggest that LSIL and HSIL are distinct biological entities, they do not clearly define their relationship. On one hand, the apparent addition of methylation events to the LSIL profile suggests that LSIL is a precursor of HSIL that requires additional cellular events, such as DAPK1 and IGSF4 methylation, for progression to HSIL. On the other hand, LSIL may be a distinct entity with a low level of DNA methylation that does not progress to HSIL.

It is interesting that the methylation profiles of anal neoplasia closely parallel those of cervical neoplasia. Hypermethylation of >20 different genes has been previously described in cervical neoplasia, most often in HSIL and invasive carcinoma (26–36). Among the most frequently methylated genes in cervical HSIL and invasive carcinoma are DAPK1, RARB, and IGSF4 (37–41). Methylation of one or more of these genes may have an antiapoptotic role in tumorigenesis (42). The frequent methylation of these genes in both the cervix and the anus suggests a common pathway, possibly related to HPV infection in these lesions. It is known that HPV proteins play a role in chromatin remodeling and transcriptional control, as does DNA methylation. Specifically, the E7 protein of HPV-16 interacts with histone acetyltransferases and deacetylases and increases the acetylation and methylation of histone H3 (43–45). One group has suggested that the expression of HPV-E7 transcripts and methylation of the CDKN2A gene are mutually exclusive mechanisms for cells to escape from the cyclin D-retinoblastoma mortality checkpoint (28); however, the exact roles of DNA methylation and HPV proteins in determining gene expression in intraepithelial lesions remain unknown.

Current screening programs for anal cancer rely on the microscopic examination of anal cytology samples for triage of patients for high-resolution anoscopy and biopsy to identify

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cytology diagnosis</th>
<th>Biopsy diagnosis</th>
<th>DAPK1 methylation</th>
<th>IGSF4 methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytology</td>
<td>Histology</td>
</tr>
<tr>
<td>1</td>
<td>LSIL</td>
<td>LSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>LSIL</td>
<td>LSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>LSIL</td>
<td>LSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>LSIL</td>
<td>HSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HSIL</td>
<td>normal</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HSIL</td>
<td>LSIL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>HSIL</td>
<td>LSIL</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>HSIL</td>
<td>LSIL</td>
<td>+</td>
<td></td>
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<tr>
<td>9</td>
<td>HSIL</td>
<td>LSIL</td>
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<td>11</td>
<td>HSIL</td>
<td>HSIL</td>
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<td></td>
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</tbody>
</table>

NOTE: +, methylation; an empty box indicates no methylation.
HSIL. Unfortunately, detection of abnormal anal cytology has a high sensitivity but low specificity for HSIL, resulting in unnecessary high-resolution anoscopy and biopsies in many patients. The identification of methylated genes that have a high sensitivity and specificity for anal and cervical HSIL raises the possibility of developing a novel cancer screening procedure using these molecular biomarkers. Several investigators have already applied such markers to cervical cytology samples (46–49). Our data suggest that DNA methylation may also serve as a useful biomarker for AIN in anal cytology samples. In addition to these potential improvements in screening programs, knowledge about DNA methylation in AIN may lead to novel therapeutic approaches using drugs that reverse DNA methylation (50).

References
DNA Methylation in Anal Intraepithelial Lesions and Anal Squamous Cell Carcinoma


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