Improved Screening for Anal Neoplasia by Immunocytochemical Detection of Minichromosome Maintenance Proteins

Cinzia Scarpini,1 Victoria White,1,2 Balaji Muralidhar,1 Aileen Patterson,3 Nicola Hickey,4 Naveena Singh,5 Jose Mullerat,6 Marc Winslet,6 R. Justin Davies,1,2 Marie-Louise Phillips,1 Pamela Stacey,1 Ronald A. Laskey,1 Richard Miller,2 Mayura Nathan,4 and Nicholas Coleman1,3

1Medical Research Council Cancer Cell Unit; Departments of 2Surgery and 3Histopathology, Addenbrooke’s Hospital, Cambridge, United Kingdom; 4Department of Sexual Health, Homerton University Hospital NHS Foundation Trust; 5Department of Histopathology, Bart’s and the London NHS Trust; and 6Department of Surgery, Royal Free and University College Medical School, London, United Kingdom

Abstract

Purpose: Early detection of anal intraepithelial neoplasia (AIN) and anal squamous cell carcinoma (SCC) by screening will improve clinical outcome. Assessment of anal cytology samples using routine Papanicolaou testing suffers from shortcomings in sensitivity and/or specificity, suggesting that screening tests based on biomarkers may be of value. We tested the suitability in this context of minichromosome maintenance (MCM) proteins, accurate markers of the deregulated cell cycle entry that characterizes malignancy and premalignancy. Experimental Design: We undertook an initial immunohistochemical study of 54 anal tissue samples and validated our findings using an independent prospective cohort study of 235 anal cytology samples from 144 subjects.

Results: In the progression from normal anal epithelium through AIN to SCC, there was increasing expression of MCM2 and MCM5, including in the superficial epithelial third, the source of the majority of cells collected by anal swab. The median labeling indices (LI) for MCM2 and MCM5 in the superficial third of AIN2/3 and SCCs combined were 90.2% and 84.0%, respectively. MCM LIs in the superficial layers were significantly greater than LIs for Ki67, an alternative marker of cell cycle entry (P < 0.0001). By immunocytochemistry using a mixture of anti-MCM2 and anti-MCM5 antibodies, immunopositive cells were readily identified in anal cytology samples, even at low magnification. MCM testing showed sensitivity for AIN2/3 of 84% (95% confidence interval, 75,93) and for AIN1/viral changes of 76% (68,84), with overall specificity (for any lesion) of 77% (64, 90).

Conclusions: MCMs are promising biomarkers for improving detection of AIN and SCC in anal cytology samples. (Cancer Epidemiol Biomarkers Prev 2008;17(10):2855–64)

Introduction

Recent years have seen a sharp increase in the incidence of anal squamous cell carcinoma (SCC; refs. 1–3), largely attributable to subpopulations of individuals at increased relative risk of developing the disease. These include men and women with HIV infection (relative risk versus general population of 33–222); men who have sex with men (MSM), either HIV positive or HIV negative; and HIV-negative women with additional risk factors (e.g., i.v. drug abuse and prostitution; ref. 4). Even before the HIV epidemic, the incidence of anal cancer was estimated to be as high as 37 per 100,000 person-years (corresponding to anal high-grade squamous intraepithelial lesion) systems, similar to squamous epithelial lesion) systems, similar to squamous cervical SCC, persistent infection with high-risk human papillomavirus (HR-HPV) is usually required for the development of AIN and anal SCC. However, the natural history of anal neoplasia is not well understood. Cross-sectional studies have shown a very high prevalence of AIN in “at-risk” groups: 41% to 97% in HIV-positive MSM; ~7% in HIV-negative MSM; 14% to 28% in HIV-positive women; and ~8% in high-risk HIV-negative women (4, 7). Moreover, four-year incidence figures for anal high-grade squamous intraepithelial lesions were 49% in HIV-positive MSM and 17% in HIV-negative MSM (11). AIN 2/3 (corresponding to anal high-grade squamous intraepithelial lesions) progressed to malignancy in 20% of 55
cases over 9 years (12). This is broadly similar to the cervix, where ~30% of cervical intraepithelial neoplasia grade 3 cases are estimated to progress to SCC over 10 years (13).

Anal liquid-based cytology has been advocated as a screening test for anal SCC and AIN (14) and may be cost-effective in MSM (7, 15, 16). Conventional Papanicolaou staining, however, even in a liquid-based platform, is subject to the same limitations of accuracy that apply to cervical cytology (14, 17) and permits only moderate intra- and inter-observer agreement (18). Due to this, alternative screening tests based on biomarkers are being considered.

HPV detection is unlikely to be useful as a primary screening test, as HPV infection is very highly prevalent in populations at risk for AIN and anal SCC, being detected by PCR in 93% of HIV-positive MSM, 60% of HIV-negative MSM, 76% of HIV-positive women, and 42% of high-risk HIV-negative women (19, 20). It is likely that greater value will be obtained from host markers of AIN/SCC. Promising candidates in detecting malignancy and premalignancy at other anatomic sites are minichromosome maintenance (MCM) proteins 2 to 7, members of the DNA prereplication complex, which are essential for DNA replication in all eukaryotic cells and for limiting replication to once per cell cycle (21, 22). All six MCMs show essentially similar distributions, being restricted to normal epithelial proliferative compartments and rapidly lost from differentiating cells (23-26). In contrast, MCM expression is altered in the proliferative deregulation that occurs in malignant/dysplastic epithelia, leading to widespread expression, including in the surface cells that are typically sampled during cytologic examination (27-29).

We previously showed that such aberrant expression of MCMs can be exploited to improve detection of cervical intraepithelial neoplasia and SCC in cervical smears (27, 30). In view of this, we examined the potential utility of MCMs as biomarkers for detecting AIN and SCC in anal cytology samples. We chose to study MCM2 and MCM5, as high-quality validated monoclonal antibodies against these proteins were available in-house. We first undertook immunohistochemical examination of the expression of MCM2 and MCM5 in normal and abnormal anal epithelium, compared with Ki67, an alternative marker of cell cycle entry. Based on our findings, we proceeded to validate the accuracy of MCM immunocytochemistry in an independent prospective cohort involving 235 anal cytology samples. Taken together, our findings suggest that immunocytochemical detection of MCMs can form the basis of an effective screening test for AIN and anal SCC.

Materials and Methods

**Tissue Samples.** Blocks of formalin-fixed, paraffin-embedded anal tissue were obtained retrospectively from the archives of the Royal Free Hospital, London, with Local Research Ethics Committee approval (Reference: 5518). We used blocks from 54 samples (each from a different patient), which represented the spectrum of histologic appearances seen in anal epithelium. The cases were chosen at random from those available in each diagnostic category. No selection criteria were applied.

The blocks used were from specimens taken between 1987 and 2001. All immunohistochemical staining was done in 2004. Histopathologic diagnosis was made according to AIN criteria (31, 32). Diagnoses were agreed by consensus between two and in many cases three consultant histopathologists, each with at least 10 years’ experience. The tissues examined represented normal anal squamous epithelium (n = 3), AIN1/viral changes (n = 20), AIN2/3 (n = 22), and SCC (n = 9). In total, 43 samples were from males, with a median age of 37 y (range 19-76 y), whereas 11 samples were from females, with a median age of 50 y (range 20-87 y). Further clinical information relating to the tissue samples was not available.

**Immunohistochemistry.** Sections of 5 μm were cut onto aminopropyltriethoxysilane-coated slides and processed for immunohistochemistry, as described previously (28, 29, 33). We used mouse monoclonal primary antibodies against MCM2 (29, 34), MCM5 (34), and Ki67 (Mib-1 clone; DAKO). Briefly, primary antibody (100 μL) was applied in a humidified chamber at 4°C overnight with gentle shaking in 1% bovine serum albumin/TBS containing 0.1% Triton X-100. The slides were then washed in TBS containing 0.025% Triton X-100 and incubated for 1 h with biotinylated goat anti-mouse secondary antibody (DAKO). A streptavidin-horseradish peroxidase system (DAKO) with the substrate diaminobenzidine was used to develop the stain and the slides were counterstained with Harris’ hematoxylin. Negative controls were done for all tissues by omitting the primary antibody.

**Quantification of Immunohistochemical Staining.** All slides were scanned using a Mirax Scan digital scanner (Carl Zeiss). For each marker, staining frequency was determined by calculating a labeling index (LI), representing the ratio of immunopositive to total epithelial cells assessed, counting a minimum of 400 cells per case. For normal anal squamous epithelium and AIN (where epithelial architecture is retained), LIs were determined for three epithelial compartments; the superficial, middle, and basal thirds of the epithelium. For each section the epithelial thirds were defined by measuring the epithelial thickness and dividing by 3. In the SCCs, epithelial orientation is lost, and we observed uniform expression of the markers studied. A single LI was therefore determined for these cases.

Counts were done manually using Analysis 3.2 software (Soft Imaging System, GmbH). All the stained slides were assessed by one observer (CGS), who had more than 15 years’ experience in analyzing immunohistochemistry slides. Interobserver variation was examined using MCM5 LIs determined by a second observer (B.M.) in 50 cases (134 counts; 36% of total counts). This observer had received in-house training in assessing immunohistochemistry slides and had over two years’ experience of doing so. Both observers were blinded to the diagnoses made by routine histopathologic examination. No clinical information was available to them.

**Statistical Analysis.** The variables of MCM2, MCM5, and Ki67 LIs are presented as medians and interquartile ranges. Correlations between the LIs of the three markers were determined using Spearman’s rank correlation coefficient, with 95% confidence intervals (CI). The
median test was used to compare differences in medians between LIs of cases where severe disease (defined as AIN2/3 and SCC) was present, and cases where it was absent (normal anus, viral changes, and AIN1). The LIs of different markers in the superficial epithelial layers were compared using the Wilcoxon signed rank test. Agreement between the MCM5 LIs determined by the two observers was examined using the 95% limits of agreement method (35). All analyses were carried out in SPSS 14 (SPSS, Inc.).

Cytology Samples for Immunocytochemistry Study. The findings from the immunohistochemistry study were validated in an independent prospective cohort study of anal cytology samples obtained from subjects attending the high resolution anoscopy (HRA) clinic at the Department of Sexual Health, Homerton Hospital, London (Local Research Ethics Committee Reference: P3/04/Q0605/27). We sought to determine the accuracy of MCM immunocytochemistry in detecting anal disease (defined as AIN1/viral changes, AIN2/3, and anal SCC; although the latter is rarely encountered in the HRA clinic). The power calculation assumed that disease prevalence in the samples obtained would be 70%, based on the mix of individuals attending the HRA clinic. We sought to establish whether sensitivity for anal disease differed significantly from 80%, and to be 80% certain that sensitivity as low as 70% would be excluded. The calculation was based on putting a CI around the sensitivity of 80% and not allowing the lower end of the CI to decrease as low as 70%, with 80% power and α = 0.05 (36). The number of samples required was 137 from patients with disease and 196 in total.

To allow for inadequate specimens, we obtained a total of 235 anal cytology samples from 144 subjects, who were recruited between November 2004 and November 2007. The first group of subjects comprised patients referred for investigation and treatment of suspected anal canal papillomas or AIN by HIV and sexual health physicians in the northeast sector of London, whereas the second comprised volunteers (generally the partners of patients) in whom there was no clinical suggestion of anal disease.

Patients were recruited into the study if they agreed to undergo anal swab examination followed by HRA and biopsy of abnormal appearing areas. Volunteers were recruited if they agreed to undergo anal swab examination followed by HRA. The volunteers were also asked to provide a tissue biopsy at the time of HRA, although this was not a requirement for entry into the study. All subjects were required to be aged ≥18 years and to understand English. There were no further inclusion/exclusion criteria. All participants were asked to declare their HIV serostatus (if known) at the time of entry into the study and all agreed to do so. Information on HIV serostatus was available for 138 of the 144 individuals in the study. The interval between the latest HIV screening test and enrollment in the study was not recorded. No additional HIV testing was done.

We invited 124 consecutive eligible subjects in the patient group to participate in the study at their first visit and 119 agreed to do, providing 119 cytology samples. Of these patients, 59 also re-attended for further investigation and treatment of anal disease. All of the re-attenders agreed to participate again in the study and they provided 72 further samples. All 191 cytology samples from the patient group were accompanied by 1 to 3 biopsies taken at HRA immediately after the anal swab was obtained. Where more than one lesion was seen, biopsies were taken from areas with differing appearances. All biopsies were fixed in 10% formalin and embedded in paraffin, followed by routine H&E staining.

Of the subjects without clinical evidence of anal disease, 29 consecutive individuals were invited to participate in the study at their first visit and 25 volunteered to do so. All of these subjects showed normal appearances at HRA. Thirteen individuals from this group also agreed to participate again in the study at subsequent visits and provided 19 further samples. In all, four volunteers agreed to provide an anal biopsy at HRA, three at their first visit and one at a follow-up appointment. Such biopsies were taken at random from the anal squamous zone. Tissue processing was as for the patient-derived samples.

Cell Sampling and Immunocytochemistry. Cells were prepared as monolayer samples by liquid-based cytology. A dry cotton bud was inserted blindly 2 cm into the anal canal and twice passed circumferentially around the anal epithelium during withdrawal, before being rinsed in Sure Path Preservative Fluid (TriPath Imaging, Inc.). The collected material was transferred to a coated microscopy slide using the Prep Stain Automated System, as per the manufacturer’s recommendations (TriPath Imaging, Inc.). The slide was then used for immunocytochemistry. No conventional Papanicolaou analysis was done, as anal cytology is not part of routine cytopathology practice in the United Kingdom and adequate expertise in interpreting Papanicolaou-stained anal samples was not available.

The immunocytochemistry staining conditions were similar to those devised previously for detection of abnormal cells in cervical smears and were designed to label at least 90% of severely dyskaryotic and malignant cells (27, 30). Immunocytochemistry was done on a DAKO Universal Stainer (DAKO). The slides were treated with 4 mmol/L sodium deoxycholate and washed in TBS. We used a mixture of purified mouse monoclonal primary antibodies against MCM2 (1:40) and MCM5 (1:20), as previous work had shown that the combination produced improved signal-to-noise ratios compared with using the antibodies individually (data not shown). The DAKO Chem Mate horseradish peroxidase detection kit was used for all subsequent steps. The slides were counterstained on a Leica Autostainer XL (Leica) using a modified Papanicolaou method, as described previously (27).

Assessment of Immunocytochemical Staining. All cytology slides were scored by a cytoscreener with over 20 years’ experience (M-LP), who was unaware of the clinico-pathologic diagnosis. Assessment criteria were derived from preliminary immunocytochemical investigations, using anal cytology samples from subjects not involved in the present study, which identified parameters that provided an optimal balance of sensitivity and specificity in detecting AIN (data not shown).

A slide was scored as positive if three or more MCM-expressing squamous epithelial cells were seen, irrespective of overall cellularity. A cell was considered to be MCM-expressing if it was immunostained using the
mixture of anti-MCM2 and anti-MCM5 antibodies. Nuclear atypia was not included in the assessment criteria, as it was found to be difficult to assess in immunopositive anal epithelial cells. If less than three MCM-expressing epithelial cells were detected, the slide was considered negative, provided at least 3,000 nucleated squamous cells were present, consistent with adequacy requirements for conventional anal cytology (37). Slides with less than three MCM-expressing epithelial cells and less than 3000 nucleated squamous cells were deemed inadequate. To assess inter-observer variation in calling a case MCM positive, MCM negative, or inadequate, 30 slides (12%) were analyzed by a second observer with over 3 years’ experience in reading immunocytochemical preparations (CGS). There was no discrepancy between the findings of the two observers.

Where possible, immunocytochemistry results were compared with the histologic diagnosis for the accompanying sampled epithelium, which served as the reference standard for the study. This information was available for 176 cytology specimens, excluding cases of inadequate cytology or histology. Histologic diagnoses were again based on the AIN system (31, 32). Diagnoses were initially made by one of the team of consultant histopathologists at Barts and the London NHS Trust, as part of their routine clinical practice. All diagnoses were subsequently reviewed by an independent consultant histopathologist with over 10 years’ experience (NS) and any discrepancy resolved by consensus. All histopathologists were provided with full clinical information relevant to each case but were blinded to the results of the immunocytochemistry study. Results were also compared with the HIV serostatus (where available) of the individuals providing the cytology samples. The accuracy of MCM2/5 immunocytochemical testing in detecting AIN was determined from the outcome measures of sensitivity and specificity values, along with their 95% CIs.

Results

Tissue Sections. In normal anal squamous epithelium, MCM2, MCM5, and Ki67 were largely restricted to the basal proliferative compartment (Fig. 1), consistent with

![Figure 1. Distribution of cell cycle markers in anal epithelium. Immunohistochemical staining showing the distribution of Ki67, MCM2, and MCM5 in representative samples of normal anal epithelium, low grade disease (AIN1), high grade disease (AIN2), and SCC. Scale bar: 200 μm.](image-url)
In the progression from normal epithelium through viral changes/AIN1 to AIN2/3, the LIs for all three markers increased in all three epithelial compartments (Fig. 2). Importantly, this increase was seen in the superficial epithelial layer, the source of the majority of cells collected in an anal cytology sample. In the SCCs, where epithelial orientation is lost, there was uniform very high expression of all the markers. The LIs in SCC were comparable with those in the basal and middle layers of AIN2/3 and greater than in the superficial layers of AIN2/3 (Fig. 2C).

The median (interquartile range) LI values in the superficial layers of severe anal disease (AIN2/3 and SCC combined, using the overall LI values for the SCCs) were 90.2 (75.9, 96.6) for MCM2, 84.0 (67.8, 89.7) for MCM5, and 62.9 (49.1, 82.8) for Ki67. For the AIN1/viral changes cases, the median (interquartile range) LI values were 49.2 (32.5, 60.8) for MCM2, 53.6 (34.2, 66.8) for MCM5, and 22.5 (7.9, 31.1) for Ki67, whereas for the normal anus samples they were 1.9 (1.5, 3.9) for MCM2, 7.5 (6.4, 8.5) for MCM5, and 1.0 (0.5, 1.0) for Ki67. For all three markers, the LI values in the superficial layers of severe disease were significantly greater than in the superficial layers of the other samples (MCM2 P = 0.002; MCM5 P = 0.0005; Ki67 P < 0.0001). In addition, the LIs in the basal and middle layers of AIN2/3 were greater than in the AIN1/viral changes and normal anus samples combined for MCM2 ( basal P = 0.002; middle P = 0.0003), MCM5 ( basal P = 0.035; middle P = 0.035), and Ki67 ( basal P = 0.0003; middle P < 0.0001).

When assessing all samples combined, we observed positive correlations among the LIs for the three markers in the superficial layers (including the overall LI values for the SCCs; Fig. 3). Spearman’s correlation coefficients were 0.672 (95% CI, 0.486-0.799; P < 0.0001; n = 51 paired readings) for MCM5 versus Ki67; 0.766 (0.573-0.878; P < 0.0001; n = 33) for MCM2 versus MCM5; and 0.751 (0.545-0.871; P = 0.001; n = 32) for MCM2 versus Ki67. The MCM2 and MCM5 LI values in the superficial layers were significantly higher than the Ki67 LIs (MCM2 versus Ki67 P < 0.0001; MCM5 versus Ki67 P < 0.0001), suggesting that MCMs would offer greater overall sensitivity than Ki67 as biomarkers for detecting abnormal cells in anal cytology samples.

Interobserver variation in the determination of MCM5 LIs was assessed using data obtained by two observers (CGS and BM) for 134 counts made on 50 samples across the range of diagnostic categories between normal anus and SCC (2 normal anus, 19 AIN1/viral changes, 21 AIN2/3, and 8 SCC). No significant differences were seen. For basal layers, the mean difference between observers (calculated as CGS – BM) was 1.32 (95% limits of agreement –6.57 to 9.22, n = 42); for middle layers the mean difference was 3.02 ( 7.11 to 13.15, n = 42); and for superficial layers (including overall LI values for the eight SCCs) the mean difference was 3.77 ( 8.39 to 15.94, n = 50).

Immunocytochemistry. The high frequency of cells expressing MCM2 and MCM5 in the superficial layers of AIN (particularly AIN2/3) and throughout anal SCCs suggested that such disease would be amenable to screening by detection of MCMs in anal cytology specimens. To validate this observation we initiated a prospective cohort study to assess the accuracy of

![Figure 2. Frequency of MCM2, MCM5, and Ki67 expression in anal lesions. Box plots showing LI values for MCM2, MCM5, and Ki67 in basal (A), middle (B), and superficial (C) layers of normal anal epithelium, viral changes/AIN1 and AIN2/3. The three columns on the right of graph C show the LIs seen throughout the SCCs, in which only single LI values were determined. Valid value frequencies for each subgroup are given in the bottom of the graphs. Bars, median; boxes, interquartile ranges; whiskers, range of data or 1.5 interquartile ranges from the end of the box; circles, outliers; stars, extreme outliers.](https://cebp.aacrjournals.org/doi/figure/10.1158/1055-9965.EPI-07-1150)
MCM2/5 immunocytochemistry in detecting AIN in anal swab samples. Details of recruitment to the study are summarized in Fig. 4.

One hundred nineteen patients attending the HRA clinic for diagnosis and treatment agreed to participate in the study. Each provided a cytology sample at his or her initial visit, which was immediately followed in all cases by HRA and biopsy of abnormal areas. All patients were treated by diode laser ablation of abnormal tissue under local anesthesia. Of this patient group, 59 also agreed to provide further samples at subsequent visits. A total of 72 samples were obtained, again all followed by HRA and tissue biopsy. For all patient samples the accompanying biopsies (where adequate) showed histologic abnormalities, with the highest grade of disease varying from viral changes (i.e., HPV-associated cytopathic effects) to AIN3.

In total, 191 cytology samples were obtained from patients with histologically confirmed anal disease. Of these, 172 proved adequate, after exclusion of 16 samples with inadequate cytology and 3 samples with inadequate histology. The 108 patients providing the adequate samples were all male, with a median age of 35 years (range 20-67 years). Of these patients, 61 were HIV seropositive, 42 were HIV seronegative, and 5 were of unknown HIV status. There were no adverse events relating to the study in the patient group.

We also recruited 25 volunteers in whom there was no clinical suggestion of anal disease. Each provided a cytology sample at his or her initial visit, which was immediately followed by HRA. Three individuals consented to anal biopsy at their first visit, whereas the remaining 22 declined. Of the volunteer group, 13 also agreed to provide further samples at subsequent visits. Nineteen samples were obtained at these follow-up visits, again all followed by HRA. One volunteer agreed to provide a biopsy at a follow-up visit, whereas the others declined. All four biopsy samples obtained from the volunteer group showed normal anal squamous epithelium.

In total, 44 cytology samples were obtained from the volunteers. 39 of these proved adequate, after exclusion of 5 samples with inadequate cytology. The 22 volunteers providing the adequate samples were all male, with a median age of 38 years (range 20-53 years). Ten of these volunteers were HIV seropositive, 11 were HIV seronegative, and 1 was of unknown HIV status. There were no adverse events relating to the study in the volunteer group.

Monolayer slides were made within 2 weeks of receipt of the cytology samples, and immunocytochemistry using a mixture of anti-MCM2 and anti-MCM5 antibodies was done 0 to 7 days later. The overall results are summarized in Fig. 4 and Supplementary Table S1. Of the 39 adequate samples from the volunteers without clinical or anoscopic evidence of anal disease, 9 (23%) were MCM positive (Fig. 5). There was no difference in HPV positivity rates in the MCM-positive samples (4 of 9) compared with the MCM-negative samples (16 of 29 where HIV status was known). No follow-up data are currently available regarding the individuals providing the MCM-positive samples. The four volunteer cytology samples with matching normal histology were all MCM negative.

MCM positivity was seen in 82 of the 108 adequate samples from patients with low grade disease (AIN1 and viral changes) and in 54 of the 64 adequate samples from patients with high grade disease (AIN2/3). In the test positive cases, immunostained cells were readily identifiable, even at low magnification. In some cases the modified Papanicolaou counterstain enabled abnormal cytologic features to be confirmed in the immunopositive cells (Fig. 5). No abnormal cells were visible by the counterstain in the 10 MCM-negative samples from patients with AIN2/3.

We calculated overall sensitivity and specificity values assuming that the biopsies taken at HRA contained the highest grade of disease in all cases and that all individuals without evidence of anal disease in whom
biopsy was not done were disease free. For all subjects combined, sensitivity for any lesion (viral changes, AIN1, AIN2, AIN3) was 79% (95% CI, 73, 85), whereas specificity was 77% (64, 90). Sensitivity for AIN2/3 was 84% (75, 93) and for AIN1/viral changes was 76% (68, 84).

Sensitivity and specificity values were also determined according to the HIV serostatus of the individuals providing the anal cytology samples (Table 1, Supplementary Table S1, and Supplementary Figs. S1 and S2). There were no significant differences, although we noted that sensitivity and specificity for any lesion in samples from HIV-seropositive subjects were greater than in samples from HIV-seronegative subjects, the increased sensitivity being due to improved detection of AIN1/viral changes (Table 1).

Finally, we reviewed the sensitivity and specificity values obtained according to the number of MCM immunopositive squamous cells required to assign a slide as test positive (Supplementary Table S2). These data confirmed our preliminary findings that three or more stained cells provided an optimal balance of sensitivity and specificity in detecting AIN. Using fewer cells (i.e., two or more or one or more) provided greater sensitivity but far less specificity, whereas using more cells (i.e., four or more) made minimal differences to sensitivity and specificity while increasing the likelihood of sample inadequacy.

Discussion

Detection of AIN or early anal SCC (<2 cm in size) would offer substantial clinical benefits. Such lesions can be treated by simple excision whereas locally advanced disease requires combined radio/chemotherapy that has many potential complications, including permanent colostomy (9%), radio/chemo toxicity (23%), and neutropenic sepsis (39). Recurrent disease post radio/chemotherapy requires extensive surgery, usually abdomino-perineal resection, and always results in a permanent colostomy (40). Overall survival is substantially affected by stage at diagnosis, with reported 5-year survival rates of 78% for local disease, 56% for regional disease, and 18% for distal disease (1).

Currently, errors and delays in the diagnosis of anal SCC are common (40). Up to 50% of patients with SCC have locally advanced disease on presentation, with a mean tumor size of 3 to 4 cm (41), emphasizing the need for improved early detection. Several groups have assessed the performance of a screening test based on cytologic assessment of cells obtained from an anal swab. Sensitivity for AIN ranged from 69% to 85% in HIV-positive subjects, but with specificity values of only 32% to 59% (14, 42-44). In a study of HIV-negative subjects, sensitivity for AIN was low at 47%, albeit with specificity of 92% (14). These shortcomings in test performance have suggested that alternative methods based on biomarkers may be of value in screening for anal disease. As well as improving accuracy and objectivity, such approaches offer the advantage of being suitable for automation, which would increase throughput and reduce costs. Host markers are likely to offer greater specificity than HPV testing, given the very high prevalence of detectable HPV in populations at risk for AIN (19, 20).

MCMs are useful markers of cell-cycle entry (22). They are abundant in the nucleus throughout the cell cycle and lost on cycle exit, with rapid loss from differentiating
cells. We have shown for a number of epithelia that whereas MCMs are normally present in basal cells only, malignancy and premalignancy are characterized by ectopic expression, frequently involving all epithelial layers, including the superficial cells (22). As it is the latter that are generally sampled in cytologic preparations (either actively, e.g., in smears or brushings, or passively by sloughing or exfoliation), immunocytochemical staining for MCMs allows discrimination between immunopositive abnormal cells and their immunonegative normal counterparts. This observation has been made for a range of epithelial types, including glandular, transitional, and squamous (22). Our observations for anal epithelium parallel those in squamous epithelia at other sites, such as the cervix (30), head and neck (29, 38), esophagus (45), and lung (46).

The greater frequency of expression of MCM2 and MCM5 compared with Ki67 in the surface layers of AIN2/3 is again consistent with studies of other epithelia (22). This observation suggests that Ki67 would detect fewer abnormal cells than MCMs in a cytologic test and therefore offer less overall sensitivity, particularly for

Table 1. Sensitivity and specificity values observed in the cytology study

<table>
<thead>
<tr>
<th></th>
<th>All adequate samples (n = 211)*</th>
<th>Adequate samples from HIV+ subjects, n = 116 (95% CI)</th>
<th>Adequate samples from HIV− subjects, n = 88 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any lesion</td>
<td>0.77 (0.64-0.90)</td>
<td>0.79 (0.61-0.97)</td>
<td>0.72 (0.52-0.93)</td>
</tr>
<tr>
<td><strong>Sensitivity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any lesion</td>
<td>0.79 (0.73-0.85)</td>
<td>0.81 (0.74-0.89)</td>
<td>0.74 (0.64-0.89)</td>
</tr>
<tr>
<td>AIN2/3</td>
<td>0.84 (0.75-0.93)</td>
<td>0.85 (0.70-0.95)</td>
<td>0.85 (0.71-0.98)</td>
</tr>
<tr>
<td>Viral changes/AIN1</td>
<td>0.76 (0.68-0.84)</td>
<td>0.81 (0.71-0.90)</td>
<td>0.68 (0.54-0.82)</td>
</tr>
</tbody>
</table>

*Seven samples were from subjects of unknown HIV status.
cases where relatively small numbers of abnormal cells are sampled. Ki67 is detectable throughout the cell cycle but unlike MCMs is only present in a fraction of cells in each phase, including cells shown to be in S-phase by in situ DNA replication (47). The function of Ki67 and its relationship to the cell cycle remain obscure.

To maximize the probability of detecting individuals with AIN2/3, our immunocytochemical staining protocol used parameters that were devised previously to stain over 90% of severely dyskaryotic and malignant cervical epithelial cells. The sensitivity values we observed were broadly similar to those seen in cervical smears (27, 30, 48). We noted greater sensitivity for disease in HIV-seropositive compared with HIV-seronegative subjects, due to increased detection of low-grade disease (AIN1/viral changes). This observation is consistent with findings using routine Papanicolaou testing of anal cytology samples (14), although comparison of sensitivities for low-grade versus high-grade disease was not made in the previous study. The 16% negativity rate that we observed in specimens from patients with AIN2/3 may be attributed to sampling error associated with blindly inserting an anal swab, as no abnormal cells could be identified on the slides in question (albeit using the limited Papanicolaou stain).

The overall specificity rate in our study of 77% was determined using samples from volunteers without clinical evidence of anal disease. Clinical investigation of these individuals was not as thorough as for patients with AIN—although all underwent HRA (with normal appearances), accompanying biopsies were only available for a small minority of the cytologic samples (4 of the 44 taken). Some individuals assumed to have normal anal epithelium may have in fact had AIN, as HRA is not able to detect all such cases (49). Further clinical information concerning the volunteers with MCM-positive cytology samples should become available in due course. Future studies will benefit from the inclusion of an adequately powered cohort of subjects in whom AIN is excluded more definitively by adequate tissue sampling at HRA. From a technical point of view, MCM—used parameters that were devised previously to reduce the percentage of AIN2/3 cells stained. Our preliminary data suggest that the number of false-positive test results would decrease based on this approach, whereas overall sensitivity would not be affected, as the number of immunopositive cells in samples from patients with AIN would still be sufficient for such specimens to be scored as test positive.

Immunocytochemical staining for biomarkers such as MCMs is well suited to liquid-based cytology platforms, particularly the Sure Path system. Slides can be read quickly and reproducibly, and multiparameter testing is possible. The ease of test interpretation renders monoclonal antibody suitable for automated slide analysis, which would produce advantages of cost and throughput. Our present data suggest that further studies validating MCM immunocytochemistry as a primary screening test for anal disease are warranted. It will also be of interest to investigate the role of MCM immunocytochemistry as an adjunctive screening test, for example in triage of anal cytologic samples showing borderline abnormalities by routine Papanicolaou testing.

Disclosure of Potential Conflicts of Interest

N. Coleman and R.A. Laskey are entitled to a share of royalties received by Cancer Research Technology Ltd on sales of products related to the use of MCM detection in cancer diagnosis.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Kalliopi Vrotsou (Centre for Applied Medical Statistics, University of Cambridge, UK) for statistical advice.

References


Unpublished data.


23. Maiorano D, Van Assendelft GB, Kearsey SE. Fission yeast cdc21, a member of the MCM protein family, is required for onset of S phase and is located in the nucleus throughout the cell cycle. EMBO J 1996;15:861–72.


Improved Screening for Anal Neoplasia by Immunocytochemical Detection of Minichromosome Maintenance Proteins

Cinzia Scarpini, Victoria White, Balaji Muralidhar, et al.


Updated version  Access the most recent version of this article at:  
http://cebp.aacrjournals.org/content/17/10/2855

Supplementary Material  Access the most recent supplemental material at:  
http://cebp.aacrjournals.org/content/suppl/2008/10/15/17.10.2855.DC1

Cited articles  This article cites 46 articles, 11 of which you can access for free at:  
http://cebp.aacrjournals.org/content/17/10/2855.full.html#ref-list-1

Citing articles  This article has been cited by 4 HighWire-hosted articles. Access the articles at:  
/content/17/10/2855.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.