Promoting early detection of HIV and anal dysplasia in Thai men who have sex with men
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CHAPTER 9

Using human papillomavirus DNA, E6/E7 mRNA, and p16 immunocytochemistry to detect and predict high-grade anal intraepithelial neoplasia in HIV-positive and HIV-negative men who have sex with men

Submitted

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Background: Men who have sex with men (MSM) are at high risk of having anal cancer. High-grade anal intraepithelial neoplasia (HGAIN) is the precursor of anal cancer. We explored the use of different biomarkers associated with human papillomavirus (HPV) infection and HPV-mediated cell transformation to detect and predict HGAIN among HIV-positive and HIV-negative MSM.

Methods: A total of 123 HIV-positive and 123 HIV-negative MSM were enrolled and followed for 12 months. High-resolution anoscopy (HRA) with biopsies were performed at every visit along with anal sample collection for cytology, high-risk HPV DNA genotyping, HPV E6/E7 mRNA, and p16 immunocytochemistry. Performance characteristics and Youden’s index (sensitivity + specificity - 1) were calculated for these biomarkers.

Results: High-risk HPV DNA, E6/E7 mRNA, and p16 immunocytochemistry each identified 43-46% of MSM whose baseline test positivity would trigger HRA referral. E6/E7 mRNA had the highest sensitivity (64.7%) and the highest Youden’s index (0.226) to detect prevalent HGAIN. High-risk HPV DNA was the most sensitive test (72.7%) to predict incident HGAIN, with the best Youden’s index (0.3).

Conclusions: Countries with a high HIV prevalence among MSM and limited HRA resources may consider using biomarkers to identify individuals at high risk of HGAIN. E6/E7 mRNA had the highest sensitivity for prevalent HGAIN detection regardless of HIV status, whereas high-risk HPV DNA performed best in predicting incident HGAIN. As none of the biomarkers evaluated in our study performed at sufficiently high level, future studies are in need to identify new biomarkers with better performance to detect HGAIN.
Introduction
Men who have sex with men (MSM) are at high risk of having anal cancer, and HIV-positive MSM have 5 times higher risk than HIV-negative MSM. High-grade anal intraepithelial neoplasia (HGAIN) is a putative precursor of anal cancer. Both the prevalence and incidence of HGAIN continue to increase in the era of highly active antiretroviral therapy (HAART) among MSM with HIV infection. Persistent anal human papillomavirus (HPV) infection, especially with high-risk types, is the most important risk factor for HGAIN and anal cancer.

Anal cytology has generally been used first in the anal intraepithelial neoplasia (AIN) screening algorithm, as it is relatively inexpensive and easy to perform. Abnormal cytology results trigger referral for high-resolution anoscopy (HRA) which permits visualization of abnormal tissue for biopsy. Once HGAIN is diagnosed, treatment is generally provided in an attempt to prevent progression to anal cancer: limited data suggests a 9-15% progression from HGAIN to anal cancer with a median follow-up of 3-5 years.

Similar to cervical cytology, low sensitivity and specificity for identifying those with biopsy-proven high-grade disease are commonly reported for anal cytology. The usefulness of other biomarkers for HGAIN screening have therefore been evaluated but the data are limited. We prospectively studied the performance characteristics of high-risk HPV DNA testing, HPV E6/E7 oncogene mRNA testing, and p16 immunocytochemistry to identify individuals with prevalent HGAIN in HIV-positive and HIV-negative MSM at an initial visit and to predict incident HGAIN in this population over 12 months.

Methods
Enrollment and follow-up of study participants
Thai men aged ≥18 years with a history of anal sex with men and documented HIV status were enrolled into the study at the Thai Red Cross AIDS Research Centre. MSM who had prior treatment for anal cancer, previous anal cytology or HRA were excluded from the study. Infrared coagulation ≤12 months before enrollment, intra-anal application of trichloroacetic acid or podophyllin ≤1 month before enrollment, or evidence of current intra-anal or perianal bacterial or herpes simplex virus anal infection at enrollment, were also exclusion criteria.

All participants gave informed consent. The study was approved by the institutional review board of Chulalongkorn University in Bangkok, Thailand (clinicaltrials.gov identification NCT01637298). Participants were followed at month-12 after baseline except for the first 120 participants who were also scheduled for month-6 follow-up. Anal sample collection, HRA and HRA-guided biopsy of visible lesions were performed at all visits by the same study physician (NT).

Anal sample collection and HRA
A moistened, non-lubricated swab (Rovers EndoCervex-Brush, Rovers Medical Devices B.V., The Netherlands, or FLOQSwabs, Copan Italia S.p.A., Italy) was used to collect anal samples. Swabs were placed into liquid-based cytology fluid (Liqui-
PREP™, LGM International, Inc., Florida, USA) and stored at 4°C for ≤7 days before processing for anal cytology and p16 immunocytochemistry. Remaining cytology fluid was stored at -80°C until processing for HPV DNA and HPV E6/E7 mRNA. HRA was performed immediately after anal sample collection, using 5% acetic acid and Lugol’s solution to aid visualization of abnormal anal tissue for biopsy.

**Diagnosis of low-grade anal intraepithelial neoplasia (LGAIN) and HGAIN**

Anal cytology results were classified using the 2001 Bethesda system as normal, atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), or carcinoma. Histologic diagnoses were classified as normal, AIN 1, AIN 2, or AIN 3. Diagnoses were given by three different pathologists and discrepancies resolved by re-evaluation, discussion, and concurrence by at least two pathologists.

A diagnosis of HGAIN was made based on a histological diagnosis of AIN 2 or AIN 3 on histology; LGAIN was AIN 1. The highest histologic grade reported was used for participants with >1 biopsy.

**HPV DNA genotyping**

HPV genotyping was done using the LINEAR ARRAY® HPV Genotyping Test (Roche Molecular Systems, Inc., Branchberg, NJ), which amplifies target DNA within the polymorphic L1 region of the HPV genome, and subsequently hybridizes this product to probes for 37 anogenital HPV DNA genotypes (13 high-risk genotypes-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 – and 24 low-risk genotypes- HPV 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108). Primers for human β-globin gene were used for quality control.

**Quantification of intracellular HPV E6/E7 mRNA**

An aliquot of liquid-based cytology fluid was used for intracellular HPV E6/E7 mRNA flow cytometry. Cell pellets were prepared, fixed and permeabilized. Fluorescence in situ hybridization for E6/E7 mRNA was performed using a cocktail of 5'- and 3'-labeled oligonucleotide probes (HPV OncoTect™ E6, E7 mRNA Kit, IncellDx, Menlo Park, CA). The kit covers the detection of E6/E7 mRNA from HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 69. Three-color flow cytometry was performed on Beckman Coulter Cytomics FC500. Cells were included in the analysis if they exhibited green fluorescence (fluorescein, HPV E6/E7 mRNA+) and blue fluorescence (4′-6-diamidino-2-phenylindole dihydrochloride, all cells) but lacked red fluorescence (polymorphonuclear cells). Samples with ≥2% of cells exhibiting E6/E7 mRNA were considered positive.

**p16 immunocytochemistry**

Slides were prepared for p16 immunocytochemistry only from samples obtained at the baseline visit by the Immunohistochemistry and Immunocytochemistry Laboratory, Department of Pathology, Chulalongkorn University. The monoclonal antibody p16INK4a was used as a primary reagent and staining was performed using
Bench Mark XT Instrument (Ventana, Medical System Inc., AZ, USA). p16 immunocytochemistry was scored by one anatomic pathologist (SK) unaware of the clinico-pathologic diagnosis and slides were considered p16-positive if cells with cytoplasmic and/or nuclear staining were present.

**Statistical Analysis**

Statistical analysis was conducted with Stata version 12.1 (Statacorp, College Station, TX, USA). The prevalence and 12-month incidence of HGAIN in individuals without HGAIN at baseline and 95% confidence intervals (95% CI) were calculated. The histologic diagnosis was used as the gold standard for comparing performance characteristics, and we assumed all participants with HGAIN were identified.

Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), negative likelihood ratio (LR), and positive LR and 95% CI were calculated for the use of anal cytology, high-risk HPV DNA, intracellular HPV E6/E7 mRNA, and p16 immunocytochemistry to detect HGAIN at baseline, for MSM overall and by HIV status. Receiver operator characteristics plots were made to demonstrate sensitivity and specificity of these tests in detecting HGAIN at baseline. Combinations of anal cytology, high-risk HPV DNA and E6/E7 mRNA to detect baseline and incident HGAIN were also evaluated. Youden's index (sensitivity + specificity -1), which describes performance when equal importance is given to sensitivity and specificity, was calculated. Performance characteristics of these tests were also studied when used at baseline and month-6 visits to predict incident HGAIN at subsequent visits, and we used Cox Proportional Hazards regression to estimate the relative risk (Hazard ratio = HR) and 95% CI of incident HGAIN in those with positive versus negative biomarker tests, for biomarkers individually and in combination, taking into account the potential of changes in performances of HRA, cytology and biomarkers over time by adjusting for chronological ordering of samples collected.

**Results**

**Participant characteristics**

A total of 246 MSM (123 each HIV-positive and HIV-negative) were enrolled from December 2009 - December 2010. Among the first 120 MSM (91 HIV-positive and 29 HIV-negative) scheduled for month-6 follow-up, 90 MSM (73 HIV-positive and 17 HIV-negative) attended the clinic. 167 MSM (89 HIV-positive and 78 HIV-negative) completed month-12 visit.

The mean (standard deviation, SD) age at enrollment was 28.8 (6.9) years for HIV-positive and 28.9 (7.4) years for HIV-negative MSM. The mean (SD) age of sexual debut was 18.0 (3.7) years for HIV-positive and 18.8 (3.7) years for HIV-negative MSM (p=0.11). In HIV-positive MSM, the mean (SD) baseline CD4 count was 353 (146) cells/mm³ and 10% had baseline plasma HIV RNA <40 copies/mL. HAART use increased from 13% at baseline to 47% at month 12. Mean (SD) CD4 count at month 12 was 388 (130) cells/mm³ and 33% had plasma HIV RNA <40 copies/mL.

**Prevalence and incidence of HGAIN**
HRA identified anal lesions in 55% (N=136/246) of participants at baseline, 66% (N=61/92) at month 6 and 56% (N=94/167) at month 12 (Table 1). Abnormal HRA findings were more common in HIV-positive MSM versus HIV-negative MSM at each visit (67% vs. 43% at baseline, p<0.001, 72% vs. 50% at month 6, p=0.08, and 68% vs. 39% at month 12, p<0.001).

Baseline prevalence of HGAIN was 18.9% in HIV-positive and 8.9% in HIV-negative MSM (p=0.03). Over the study period, 28.8% of HIV-positive and 4.1% of HIV-negative MSM with no HGAIN at baseline, developed HGAIN.

Baseline anal cytology, high-risk HPV DNA, E6/E7 mRNA, and p16 immunocytochemistry

**Anal cytology**

Abnormal anal cytology defined as ASC-US or worse was found in 12.7% (11.2% of HIV-positive and 14.1% of HIV-negative) MSM at baseline (Table 2). MSM without AIN had lower rates of abnormal anal cytology (0.8%) compared with those with LGAIN (29.5%) and HGAIN (18.8%), p<0.001. The same significant trend was seen in HIV-positive (p=0.001) and HIV-negative MSM (p<0.001).

**High-risk HPV DNA genotyping**

High-risk HPV types were detected in 46.3% of MSM at baseline (56.1% of HIV-positive and 36.5% of HIV-negative MSM) (Table 2). HPV 16 and/or 18 infections were detected in 20.3% of MSM (27.5% of HIV-positive and 13% of HIV-negative
were MSM. High-risk HPV types were identified in 39.1% of MSM without AIN, 57% of MSM with LGAIN and 50% of MSM with HGAIN (p=0.05). HPV 16 and/or 18 infections were detected in 13.6% of MSM without AIN, 29.8% of MSM with LGAIN and 23.5% of MSM with HGAIN (p=0.03).

**E6/E7 mRNA**
Baseline E6/E7 mRNA was positive in 45.5% of MSM (49.2% of HIV-positive and 41.8% of HIV-negative MSM) (Table 2). E6/E7 mRNA positivity increased from 35.6% of MSM without AIN to 53.8% of those with LGAIN and 64.7% of those with HGAIN (p<0.001). The same trend was seen in HIV-positive MSM (p=0.005).

**p16 immunocytochemistry**
Positive p16 immunocytochemistry was found among 43.4% of MSM at baseline (41.9% of HIV-positive and 45% of HIV-negative MSM) (Table 2). There was no trend in positivity rates of p16 immunocytochemistry with increasing grades of AIN.

**Performance characteristics of individual biomarkers to detect baseline HGAIN**
Among all MSM at baseline, anal cytology had a sensitivity of 18.8% and a specificity of 88.2% to detect HGAIN. E6/E7 mRNA was the most sensitive test (64.7%), followed by high-risk HPV DNA (50%), p16 immunocytochemistry (31.3%), and HPV 16 and/or 18 (23.5%) (Table 3). Specificity was approximately the same for E6/E7 mRNA (57.9%), high-risk HPV DNA (54%), and p16 immunocytochemistry (56.5%). Youden's index was highest for E6/E7 mRNA (0.226), indicating the highest proportion of correctly classified cases. With the exception of this biomarker, trends in individual biomarker performance were similar in all MSM and HIV-positive MSM: a negative Youden's index seen in HIV-negative MSM indicates a greater proportion of negative test results in those with HGAIN than those without (Table 3). Figure 1A demonstrates the performance characteristics of each test in detecting baseline HGAIN. (Table 3). The overall PPVs of these tests were low and ranged from 11.1% to 20%. The PPVs tended to be higher in HIV-positive (ranged from 18.2% to 38.5%) than HIV-negative MSM (ranged from 2.4% to 11.8%). p16 immunocytochemistry had the lowest PPV while E6/E7 mRNA had the highest PPV, compared with the other tests.

**Performance characteristics of biomarkers to predict incident HGAIN**
Among MSM without HGAIN at baseline, a single positive high-risk HPV DNA test result either at the baseline or month-6 visit was the most sensitive test to predict incident HGAIN at a subsequent visit (72.7%), followed by p16 immunocytochemistry (58.3%) and E6/E7 mRNA (46.2%) (Table 4). Of these three tests, high-risk HPV DNA also had the highest specificity (57.3%), followed by p16 immunocytochemistry (47%) and E6/E7 mRNA (43.5%). Youden's index was best for high-risk HPV DNA (0.3). Considering positive test results at two consecutive time points (at both baseline and month 6 visits), persistent detection of high-risk HPV DNA was able to predict HGAIN at a subsequent visit with the highest sensitivity (54.5%) and the highest Youden's index of 0.205. This was supported by a proportional hazards analysis adjusting for the chronological ordering of the tests throughout the study, in which having positive high-risk HPV DNA at two consecutive time points was
associated with the highest relative risk of incident HGAIN (HR 3.31, 95% CI 1.38-7.97, p=0.01, data not shown). Compared with the other tests, anal cytology had the highest PPVs when a single positive test (22.7%) and two consecutive positive tests (40%) were used to predict incident HGAIN.

**Performance characteristics of biomarkers when used in combination**

HPV DNA used in combination with E6/E7 mRNA to detect prevalent HGAIN showed an overall higher sensitivity (82.4%), but lower specificity (32.9%) than the use of either test alone (Table 3, Figure 1B). Youden's index (0.153) and the PPV (16.6%) of this combination were higher than high-risk HPV DNA but lower than E6/E7 mRNA.
used alone. Youden's index for anal cytology used with E6/E7 mRNA (0.271) was highest among the combinations, and was higher than E6/E7 mRNA alone.

For incident HGAIN, anal cytology and high-risk HPV DNA in combination provided a higher Youden's index than other combinations, both when the test was positive at a single or two consecutive time points. Youden's index was also higher with this combination than with high-risk HPV DNA alone.

![Table 3](attachment:table3.png)
Discussion

The high prevalence (18.9%) and 12-month incidence (28.8%) of HGAIN observed among HIV-positive MSM in our study were within the range reported in Western studies.\(^5,7,9,23,24\) Together, these data demonstrate the critical importance of implementing HGAIN screening programs where HIV is common among MSM. In both resource-limited and resource-rich countries, there is a paucity of HRA equipment and clinicians trained in HRA. Therefore, biomarkers could potentially identify those with the highest risk of HGAIN, who would benefit most from HRA referral.

**FIGURE 1.** Receiver operator characteristics plots of anal cytology, high-risk HPV DNA, HPV 16 and/or 18, E6/E7 mRNA, and p16 immunocytochemistry performances to detect high-grade anal intraepithelial neoplasia (HGAIN). (A) Performance characteristics when each test was used alone to detect HGAIN. (B) Performance characteristics when tests were used in combination to detect HGAIN.

HGAIN, high-grade anal intraepithelial neoplasia.

HGAIN included anal intraepithelial neoplasia (AIN) 2 or AIN 3 on histology.
Performance characteristics of biomarkers associated with anal HPV infection and HPV-mediated cell transformation demonstrated that high-risk HPV DNA, E6/E7 mRNA, and p16 immunocytochemistry, when used as a primary screening test to trigger referral of MSM at high risk for HGAIN to HRA, identified comparable proportions (ranging from 43-46%) of men with baseline positivity. E6/E7 mRNA had higher sensitivity, higher PPV, and higher Youden's index than the other tests. Anal cytology only identified 12.7% of MSM for HRA referral with a lower sensitivity and lower Youden's index than E6/E7 mRNA, although with comparable PPV. Use of E6/E7 mRNA in combination with anal cytology provided a higher sensitivity and a higher Youden's index than when either test was used alone.

Recent studies have compared the performances of E6/E7 mRNA and high-risk HPV DNA to detect HGAIN among HIV-positive MSM. A US study demonstrated that E6/E7 mRNA provided a lower sensitivity than high-risk HPV DNA (79.8% vs. 100%) but with a higher Youden's index (0.422 vs. 0.277) and PPV (50.9% vs. 40.3%) to detect HGAIN. Baseline E6/E7 mRNA positivity was 49.3%, and 79.1% for high-risk HPV DNA. In a German study, E6/E7 mRNA demonstrated similar sensitivity to high-risk HPV DNA but with a two-fold higher specificity (46.0% vs. 26.1%) and a higher...
PPV (25.2% vs. 19.7%).\textsuperscript{20} E6/E7 mRNA was detected in 60.6% and high-risk HPV DNA was detected in 77.3% of MSM in this study. Although direct comparisons with HIV-positive MSM in our study are limited by an approximately 20-year age gap and different laboratory techniques, the data agree insofar as none of these tests performed well to detect HGAIN. E6/E7 mRNA, however, had a higher Youden's Index and PPV than high-risk HPV DNA, and resulted in less number of HRA referral. This is potentially useful for HGAIN screening programs where HRA services are limited.

For predicting incident HGAIN among those with no HGAIN at baseline, high-risk HPV DNA performed best, while E6/E7 mRNA and p16 immunocytochemistry, which are considered biomarkers that identify transforming HPV infections and precancer, did not perform as well. Types of high-risk HPV covered by these tests are unlikely to explain this finding as the high-risk HPV types covered by the HPV DNA genotyping test and the E6/E7 mRNA kit used in our study are almost identical and p16 immunocytochemistry is not type-restricted. Nevertheless, because of our small number of incident cases these data should be interpreted with caution.

HRA performance on all study participants and rigorous anal sampling for different biomarkers by the same study physician is a major strength of our study. However there are several limitations. A lower biopsy rate and low positivity of anal cytology compared with previous studies might indicate that some cases, later included as incident cases were missed at baseline.\textsuperscript{5,25} Such a scenario resulting in performance changes of HRA and anal cytology over the study period may affect the overall performance characteristics of the studied biomarkers to detect prevalent and incident HGAIN, but this is likely minimized by the 12-month enrollment period which resulted in some HRA and biomarkers at initial visits being performed at the same time as some month 12 visits. Similarly, our inclusion of month-6 data from a subset of participants might also result in an overestimation of the overall sensitivity of biomarkers to detect incident HGAIN. Nonetheless, long-term follow-up of this cohort will further strengthen the understanding of biomarkers used to detect HGAIN among HIV-positive and HIV-negative MSM.

In summary, we demonstrated that the use of E6/E7 mRNA correlated best with prevalent HGAIN detection both among HIV-positive and HIV-negative MSM. For predicting development of incident HGAIN, high-risk HPV DNA performed better than other biomarkers studied. Countries with high prevalence of HIV infection among MSM and limited resources for HRA may benefit from using biomarkers in their HGAIN screening programs for MSM. As none of the biomarkers evaluated in our study performed at high level, additional studies are in need to identify new biomarkers or combinations of biomarkers with better performances to detect HGAIN.

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