Anal intraepithelial neoplasia in HIV+ men
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One lesion, one virus: Individual components of High Grade Anal Intraepithelial Neoplasia in HIV+ men contain a single HPV type

Olivier Richel, Koen D. Quint, Jan Lindeman, Carel J.M van Noesel, Maurits N.C. De Koning, Henk A.M. van den Munckhof, Henry J.C. de Vries, Jan M. Prins, Wim G.W. Quint

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Abstract

Background High-grade anal intraepithelial neoplasia (HGAIN) is present in many HIV+ men-who-have-sex-with-men (MSM). The major etiologic factor is infection with an oncogenic human papilloma virus (HPV). We investigated whether individual components of HGAIN are caused by single HPV types.

Methods DNA was isolated from 31 HGAIN whole tissue sections (WTS) of 21 HIV+ MSM and analysed by the SPF10 PCR/LiPA25 HPV genotyping system. In WTS with multiple HPV types, PCR was repeated in regions of AIN sampled by laser-capture-microdissection (LCM). The results were compared with HPV types in anal swabs.

Results A single HPV type was observed in 15 (48%) of 31 WTS. In an additional 14 WTS, one HPV type was found in each LCM lesion sample. Consequently, in 29/31 (94%) biopsies a single HPV type was found in each lesional component studied. 2 WTS contained collision regions with each 2 HPV types. HPV 16 was presumed causative in 14/31 biopsies (45%). More than half of the anal swabs did not contain all causative HPV types.

Conclusions Individual components of HGAIN are caused by single HPV types (“one lesion, one virus”). HPV 16 is causative in less than 50%. Anal swabs are not useful for detecting lesion specific HPV types.

Keywords: anal intraepithelial neoplasia, anal cancer, human papillomavirus, human immunodeficiency virus, laser capture microdissection
Introduction

The incidence of anal cancer among HIV+ men who have sex with men (MSM) is increasing. Incidence rates exceed those of cervical cancer before the introduction of cervical screening programs.\(^1\)\(^2\) Like cervical cancer, anal cancer is believed to arise from a squamous precursor, called anal intraepithelial neoplasia (AIN), and has been causally linked to infection with oncogenic human papillomavirus (HPV).\(^3\)\(^5\)

Given the successful HPV-vaccination program in women to prevent cervical cancer, prophylactic vaccination to prevent anal cancer is under investigation. Prophylactic HPV vaccination was shown to be effective in preventing AIN among young HIV negative MSM, reduced recurrence of high grade (HG)AIN in HIV negative men, and was also highly immunogenic in HIV-1 infected men.\(^6\)\(^7\)\(^8\) Therapeutic vaccination, aiming at strengthening the HPV-specific T cell response, is also a subject of discussion.\(^9\) For both prophylactic and therapeutic vaccination it is essential to know which HPV types cause AIN and anal cancer. Furthermore, in AIN screening programs it could be valuable if the malignant potential of an AIN lesion were assessed through determination of the causative HPV type.

Previously it has been shown that HPV 16 is present in the majority of anal cancer and HGAIN lesions.\(^10\)\(^11\) However, in anal swabs obtained from of HIV+ patients who are the highest risk group, HPV16 is less common and often multiple HPV-genotypes are found.\(^12\)\(^13\) The question is whether it is possible to link a specific HPV type to the HGAIN lesion in cases with multiple HPV genotypes in a swab.

Laser-capture micro-dissection (LCM) combined with sensitive and type-specific PCR has proved to be a reliable method of finding the lesion-specific HPV genotype in cervical and vulvar (VIN) intraepithelial neoplasia.\(^14\)\(^16\) We recently demonstrated that every CIN lesion or every individual component of a CIN lesion contains one specific HPV type.\(^15\) This study also showed that in 94% of the whole tissue sections (WTS) containing a single HPV type, the same HPV type was found by LCM in the dysplastic area within the WTS. When multiple HPV types were present by WTS-PCR, the LCM-PCR technique was found to be very accurate for high-resolution HPV genotyping and for assigning an individual
HPV type to an area of CIN, resulting in the concept of “one lesion, one virus”. In the present study, we investigated the hypothesis that individual components of HGAIN are associated with one single HPV type in HIV+ MSM. Secondary goals were to survey the spectrum of HPV types responsible for HGAIN in this high-risk group and to compare HPV types in swabs, WTS and LCM-selected regions with each other.

Materials and methods

Patients
This study is a substudy of a larger trial on AIN treatment at the HIV outpatient clinic and department of Dermatology of the Academic Medical Center (AMC) in Amsterdam, where a screening program for anal precancer is in place since 2008. The study was approved by the local ethical committee and all patients gave written informed consent. Patients were screened by high-resolution anoscopy (HRA) with biopsies of suspect lesions for histopathological analysis. Twenty-one patients with histologically proven HGAIN (AIN 2 or 3) were included in the present sub-study. All biopsies were taken during HRA and in the majority of patients anal swabs (Dacron) were taken for HPV analysis within 4 weeks after biopsy. Selection of patients took place without knowledge of HPV status. The swabs were collected retrospectively and not all swabs were taken on the same day as the biopsy (see the result section for further details).

Pathological diagnosis and grading
All biopsies were examined independently by two pathologists (CVN and JL). Diagnosis of AIN was made according to standard criteria on haematoxylin and eosin (H&E)-stained sections (CVN). P16 immunohistochemistry (p16 IHC) was used to support the diagnosis of HGAIN. The overall diagnosis was the highest grade detected in a lesion. Biopsies could also include areas of low grade (LG) AIN and normal anal mucosa.

After routine diagnosis by the pathology department of the AMC, the slides, blocks and swabs were sent to DDL Diagnostic Laboratory for the second examination (JL) and further molecular analyses.
Sandwich cutting
All biopsy blocks were sectioned according to the sandwich cutting procedure as described previously.15 A 4 µm section for HGAIN diagnosis (H&E before); two sets of 3 x 4 µm for whole tissue section (WTS)-PCR analysis, 2 x 4 µm sections for LCM (collected on PEN-membrane slides from Carl Zeiss B.V., Sliedrecht, The Netherlands), then 2 x 4 µm sections for p16 IHC, and finally, a 4 µm section for pathological confirmation (H&E after). After sectioning each block, the microtome was cleaned and a new knife was used for the next block. The haematoxylin stained PEN-membrane slides were used for LCM PCR when multiple HPV types were found in the WTS-PCR. Negative controls (paraffin blocks) were used after every ten blocks sectioned to check for cross-contamination.

Laser Capture Micro-dissection
When multiple HPV genotypes were detected in the WTS-PCR, LCM was used to obtain discrete areas of dysplastic anal epithelium from HGAIN lesions. As a control, four biopsies with a single HPV type were analyzed. Slides were scanned using digital microscopy (Aperio Technologies Inc, Vista, CA, USA). An expert pathologist (JL) selected areas of AIN for sampling. A minimum of one LCM sample was taken of each HGAIN lesion, covering a representative part of the lesional area. Sample size was between 12,000 and 1,000,000 µm². Selected regions were excised with Zeiss P.A.L.M. microbeam UV laser micro-dissection system and transferred to an AdhesiveCap500 opaque tube (Carl Zeiss B.V., Sliedrecht, The Netherlands). In addition, LCM was performed on a negative control block (human placenta) for each case examined. DNA isolation and PCR for HPV DNA were performed as described below. An example of LCM is shown in figure 1.

DNA isolation
Total DNA was isolated from formalin-fixed paraffin-embedded material by proteinase K digestion.19 Briefly the tissue sample was added to 100 µl of proteinase K lysis buffer and incubated at 70 °C for 16-24 h. Proteinase K was heat-inactivated by incubation at 95 °C for 10 min. Each DNA isolation run contained HPV positive and negative controls.

The DNA isolation in swabs was performed using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany), including pre-treatment with proteinase K, according to the manufacturer’s instructions.
**HPV DNA detection and genotyping**

Ten microlitres of isolated DNA were added to 40 µl of PCR mix. The SPF$_{10}$ PCR primer set amplifies a small fragment of 65 bp from the L1 region of mucosal HPV genotypes. Amplification products (HPV DNA) were detected using the HPV SPF$_{10}$ PCR (version 1) DNA enzyme immunoassay (DEIA). DEIA positive amplimers were used to identify the HPV genotype by reverse hybridisation with the HPV line probe assay (LiPA$_{25}$), containing probes for 25 most common mucosal HPV genotypes [HPV genotypes 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74; SPF10 HPV LiPA25 version 1 (Labo Biomedical Products, Rijswijk, The Netherlands, based on licensed Innogenetics technology)].

PCR products were also tested with an in-house extended RHA strip, additionally detecting less common mucosal HPV types 26, 30, 55, 61, 62, 64, 67, 69, 71, 82-85, 87, 89-91, as described previously. HPV55 and HPV64 are now classified as subtypes of HPV44 and HPV34, respectively.

**Results**

**Participants and biopsies**

We studied 31 HGAIN (AIN 2/3) lesions obtained from 21 HIV+ MSM. The median age of the participants was 48 years (interquartile range (IQR) 41-60) and they were known to be HIV positive for a median of 11 years (IQR 5-16). The current, median CD4 cell count was 480 cells/µl (IQR 350-665) and 95% were using antiretroviral therapy. For patients with multiple biopsies, the biopsies were taken from distinct macroscopic lesions. In 12 patients one biopsy was taken, in 8 patients two biopsies and in one patient three biopsies.

**Whole tissue sections**

HPV was detected in all 31 (100%) WTS. HPV genotyping analysis of WTS of the 31 biopsies showed a single HPV genotype in 15 (48%) WTS and multiple HPV types in 16 (52%) WTS, as shown in figure 2. The median number of HPV types per WTS was 2 (range 1-7). In total 20 different HPV genotypes were found. The predominant HPV type in the WTS was HPV 16 (present in 16/31), followed by HPV 31 (6/31) and HPV 18 and 51 (both present in 4/31). Further details are presented in table 1.
**Figure 2** Single vs. multiple HPV types in the 31 HGAIN biopsies

In the 16 WTS containing more than one HPV type, 53 dysplastic regions (including LGAIN (AIN 1) regions) were selected by LCM and analysed. The median number of captured dysplastic regions per WTS was 3 (range 2-6). In 14/16 WTS with more than one HPV type a single HPV type was found in each LCM-selected region: in 10 only one HPV type was found in all regions and in 4 WTS different regions of the lesions contained different HPV types. Two WTS with more than one HPV type contained two HPV types in a single LCM-region. These were HPV 16 and 26 (biopsy no. B5) and 11 and 31 (biopsy no. B8) (table 1, figure 3 and figure 4).

In 15/16 WTS, all HPV types found in the LCM regions were also present in the WTS/PCR. One biopsy had a lesion specific HPV type that was not seen in the WTS/PCR. It was an HPV of undetermined type (X-type) (biopsy B22).

**Laser-capture micro-dissection**

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Figure 3 Multiple HPV types in two regions

H-stained membrane slides. Two, possibly colliding, regions containing two HPV genotypes. A: LGAIN with HPV 16 and 26 (biopsy ID 5). B: HGAIN with HPV 11 and 31 (biopsy ID 8).

Figure 4 Whole tissue section with a collision region

Biopsy ID 5 (see table 1) with a collision lesion containing both HPV 16 and 26. Both were also found in separate regions. HPV 31 was found in another separate region.

Figure 5 Two non-dysplastic control lesions containing HPV 74.

Biopsy ID 31 in table 1.
As controls, we performed LCM in four WTS containing only one HPV type (biopsy no. B14, B16, B17 and B20). All LCM regions contained the same single HPV type as observed in the WTS (table 1).

Furthermore, in 11 WTS, LCM was used to select 15 non-dysplastic regions, of which 10 were HPV negative. Four non-dysplastic regions showed the same HPV type as adjacent dysplastic regions (as shown in figure 5 for biopsy no. B31). One non-dysplastic region contained a HPV type (HPV 91) that was not present in the adjacent dysplastic regions.

**HPV detected in lesions: combined result of WTS and LCM**

In summary, 15 WTS contained a single HPV type, and 16 WTS contained multiple HPV types. In 10 of those, LCM selected lesional regions contained the same HPV type in all dysplastic regions of the WTS. Therefore, 25 (15+10) of 31 AIN lesions were associated with a single HPV type. In addition, 4 WTS showed more than one HPV type, but all restricted to a single region within the WTS, adding up to a total of 29/31 biopsies (94%) in which a single HPV type was found either on its own in the WTS or in a separate component of the HGAIN lesion. The remaining two showed 2 HPV types in a single LCM selected region (figure 3), although the surrounding lesional regions showed single HPV infections with each of the types (figure 4). The regions with two HPV types are therefore collision regions of two separate single HPV infection regions.

In the WTS we found 20 different HPV types, whereas in total 17 different lesional HPV types were found. HPV 16 was a lesional HPV type in 14/31 (45%), followed by HPV 18, 31 and 58 (all 3 present in 3 lesions) (table 1).

**HPV analysis of anal swabs**

Anal swabs for HPV analysis were available for 15 patients: in 6 the anal swabs were taken on the same day as the biopsy, in 9 participants the swab was taken within 4 weeks after biopsies. In total 28 different HPV genotypes were found. The median number of HPV types per swab was 4 (range 1-13). The predominant HPV type was HPV 16 (present in 7/15 swabs), followed by HPV 18, 39, 51, 52 and 70 (all present in 4/15 swabs) (table 1).

Eight of the 15 anal swabs did not contain all the lesional HPV types found in the WTS or selected LCM areas. For patients with anal swabs taken on the same day, 3/6 swabs did not contain all lesional types.
Table 1 31 anal biopsies in 21 HIV+ men, with HPV genotyping results of anal swabs, whole tissue sections (WTS) and laser-capture micro-dissection (LCM) selected regions.

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LCM was performed in the 16 WTS containing multiple HPV types and in 4 WTS containing one HPV type (controls). A HGAIN biopsy occasionally contained both HGAIN and LGAIN regions. *Swab taken on the same day as biopsy. †One HPV type could not be determined. ‡Collision lesions. §The combined HPV-results of WTS and LCM.

**Discussion**

In this study we performed HPV genotyping on WTS and LCM selected regions of HGAIN lesions in HIV+ MSM. We were able to identify the single and presumably causative HPV types in all regions of HGAIN lesions, although 2 LCM-PCR samples contained a collision region. We showed that analysis of WTS is not sufficient to determine the causative HPV type if multiple HPV types are present, and anal swabs often do not contain the causative HPV type. Finally, we found that almost half of the lesions are not caused by the two oncogenic HPV types (HPV 16 and 18) targeted by current prophylactic vaccines.

These results cast doubt on the biological significance of HPV genotypes in anal swabs in relation to the question which HPV type is causing the AIN. In previous studies the vast majority of anal swabs taken in HIV+ MSM showed multiple HPV types.23, 24 Our study shows similar data in a group with confirmed HGAIN and in addition we found that more than half of the anal swabs did not contain the HPV type present in the lesion. Unfortunately, most swabs were not taken on the same day as the biopsy. However, we do not consider that this influenced
our findings since the HGAIN lesions had not yet been treated between the time of biopsy and swab collection, and swabs taken on the same day showed the same proportion of lesional HPV types not present in the swab.

Although our numbers are small, anal swabs are possibly not useful in HIV+ MSM in predicting (HG) AIN or detecting the causative HPV type. Given the high number of (oncogenic) HPV types in the anal canal in HIV+ MSM and the possible presence of a lot of “passenger” or “transient” infections, the ability to identify the causative type might be lower than in cervical screening programs in HIV negative women. In our study we used a very sensitive SPF10/DEIA/LiPA25 (version1) technology to identify the HPV types present (high analytical sensitivity). In general, very low levels of HPV do not reflect a clinically meaningful infection i.e. an infection associated with CIN3+ or cervical cancer, but rather a transient or latent infection. To investigate whether anal HPV screening could be relevant, we think a less sensitive HPV-test should be used. Testing with a too sensitive test will result in lower specificity and a low positive predictive value, that is, the diagnosis of more irrelevant infections, which could result in an excess of follow-up tests, referrals and treatment. On the other hand, despite the use of this very sensitive technology, testing of anal swabs had still insufficient sensitivity to detect the causative HPV type.

HPV genotyping of whole tissue sections of biopsies has also been used to identify HPV causing a lesion. However, it has previously been shown that HGAIN biopsies often contain more than one HPV type. The question is whether all HPV types found in a WTS play a role in the dysplasia or that some are a casual infection of the lesion in the mucosa or have been deposited on the epithelial surface. HPV genotyping of LCM-selected areas of CIN lesions was shown to eliminate irrelevant HPV types. In the current study we established that in case of multiple HPV types observed in WTS, a single HPV type was found in each component of the HGAIN lesion in 29 of the 31 cases. In addition, the two lesions with a double HPV infection in an LCM sample appeared to represent collision regions between two lesions each infected with a single HPV type. This confirms that the “one lesion, one virus” theory previously proven for VIN and CIN lesions in immunocompetent women, is also valid for AIN lesions in HIV+ MSM.
In addition to the dysplastic areas, we found that five of 15 LCM-selected non-dysplastic areas were HPV positive. Four of those showed the same HPV type as adjacent dysplastic areas in the same tissue section. In one non-dysplastic area we found an HPV type which was not present in the dysplastic LCM areas in the same tissues section. From our cervical experience we also see HPV DNA in normal epithelium in a small proportion of cases. We have evidence that this form of “latency” occurs in resolving HPV infections and we would expect it not to be uncommon in HIV+ men.

Both AIN and anal cancer are far more prevalent in HIV+ MSM than CIN and cervical cancer in immunocompetent women and HGAIN in HIV+ MSM is more often caused by non-HPV16/18 types. More information is needed on the oncogenic capacity of these HPV types. If the pattern of genotype distribution found here in HGAIN is seen in anal cancer, vaccination programs with the current available bivalent and quadrivalent HPV vaccine might not be sufficient to prevent anal cancer in this important risk group. A recent study showed that 55% of 52 anal cancer specimens in HIV+ patients contained multiple HPV types. In the same study, 26/52 contained a non-vaccine HR HPV type. Two smaller studies show a comparable proportion of non-vaccine HPV types in anal cancers of HIV+ patients. Future multivalent HPV vaccinations, like the nona-valent vaccine currently evaluated in clinical trials, might be more appropriate for preventive programmes focused on anal cancer.

Studies are needed of the relationship between HPV types in lesions and progression from low to high grade AIN and anal cancer. In AIN screening programs it would be of value to discriminate between low-risk and high-risk HGAIN lesions based on the malignant potential of the specific HPV type in the lesion. For these purposes HPV genotyping of WTS and, in the case of multiple HPV types, of LCM selected regions is needed.

In conclusion, almost each morphologically distinct area of HGAIN can be attributed to a single HPV type, as previously seen in CIN and VIN. With sensitive PCR for HPV on WTS and LCM-selected regions the lesional HPV type can be identified. The prevalence of HGAIN is very high among HIV+ MSM, and future studies should focus on the malignant potential of different HPV types in order to target AIN prevention and treatment more efficiently.
References


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