One Lesion, One Virus: Individual Components of High-Grade Anal Intraepithelial Neoplasia in HIV-Positive Men Contain a Single HPV Type

Olivier Richel,1 Koen D. Quint,5,6 Jan Lindeman,2 Carel J. M. van Noesel,2 Maurits N. C. De Koning,5 Henk A. M. van den Munckhof,5 Henry J. C. De Vries,3,4 Jan M. Prins,1 and Wim G. V. Quint5

1Division of Infectious Diseases, Department of Internal Medicine, 2Department of Pathology, and 3Department of Dermatology, Academic Medical Center, and 4STI Outpatient Clinic, Cluster for Infectious Diseases, Public Health Service Amsterdam, Amsterdam, 5DDL Diagnostic Laboratory, Rijswijk, and 6Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands

Background. High-grade anal intraepithelial neoplasia (AIN) is present in many human immunodeficiency virus (HIV)–positive men who have sex with men. The major etiologic factor is infection with an oncogenic human papillomavirus (HPV) genotype. We investigated whether individual components of high-grade AIN are caused by single HPV types.

Methods. DNA was isolated from whole-tissue sections of 31 high-grade AIN that were recovered from 21 HIV-positive men who have sex with men. The SPF10 PCR/LiPA25 HPV genotyping system was used for DNA analysis. In whole-tissue sections with multiple HPV types, polymerase chain reaction was repeated in regions of AIN sampled by laser-capture microdissection. The results were compared with HPV types in anal swabs.

Results. A single HPV type was observed in 15 (48%) of 31 whole-tissue sections. In an additional 14 whole-tissue sections, 1 HPV type was found in each lesion sample evaluated by laser-capture microdissection. Consequently, in 29 of 31 biopsy specimens (94%), a single HPV type was found in each lesional component studied. Two whole-tissue sections contained collision regions, each with 2 HPV types. HPV16 was presumed to be causative in 14 of 31 biopsy specimens (45%). More than half of the anal swabs did not contain all causative HPV types.

Conclusions. Individual components of high-grade AIN are caused by single HPV types (the so-called one lesion, one virus concept). HPV16 is causative in <50% of cases. 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.

The incidence of anal cancer among human immunodeficiency virus (HIV)–positive men who have sex with men 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 success of HPV vaccines in the prevention of cervical cancer, prophylactic vaccination to prevent anal cancer is under investigation. Prophylactic HPV vaccination was effective in preventing AIN among young HIV-negative men who have sex with men, had reduced recurrence of high-grade AIN (defined as AIN grade 2 or 3) in HIV-negative men, and was also highly immunogenic in HIV type 1–infected men [6–8]. Therapeutic vaccination, aimed 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 HPV16 is present in the majority of anal cancers and high-grade AIN lesions [10, 11]. However, in anal swabs obtained from HIV-positive patients, who are the highest risk group, HPV16 is less common, and multiple HPV genotypes are often found [12,13]. The question is whether it is possible to link a specific HPV type to the high-grade AIN lesion in cases with multiple HPV genotypes in a swab.

Laser-capture microdissection (LCM) combined with sensitive and type-specific polymerase chain reaction (PCR) has proved to be a reliable method of finding the lesion-specific HPV genotype in cervical intraepithelial neoplasia (CIN) and vulvar intraepithelial neoplasia (VIN) [14–16]. We recently demonstrated that every CIN lesion or every individual component of a CIN lesion contains 1 specific HPV type [15]. This study also showed that in 94% of the whole-tissue sections containing a single HPV type, the same HPV type was found by LCM in the dysplastic area within the whole-tissue sections. When multiple HPV types were detected by PCR of whole-tissue sections, 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 so-called one lesion, one virus concept.

In the present study, we investigated the hypothesis that individual components of high-grade AIN are associated with a single HPV type in HIV-positive men who have sex with men. Secondary goals were to survey the spectrum of HPV types responsible for high-grade AIN in this high-risk group and to compare HPV types in swabs, whole-tissue sections, 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 in Amsterdam, where a screening program for anal precancer has been in place since 2008. The study was approved by the local ethics committee, and all patients gave written informed consent [17]. Patients were screened by high-resolution anoscopy, with biopsy specimens from suspect lesions obtained for histopathologic analysis [17].

Twenty-one patients with histologically proven high-grade AIN were included in the present substudy. All biopsy specimens were taken on the same day as the biopsy (see the result section for further details).

**Pathological Diagnosis and Grading**

All biopsy specimens were examined independently by 2 pathologists (C. J. M. v. N. and J. L.). Diagnosis of AIN was made according to standard criteria by analyzing hematoxylin and eosin–stained sections (performed by C. J. M. v. N.) [18]. P16 immunohistochemistry analysis was used to support the diagnosis of high-grade AIN. The overall diagnosis was the highest grade detected in a lesion. Biopsy specimens could also include areas of low-grade AIN (defined as AIN grade 1) and normal anal mucosa.

After routine diagnosis by the pathology department of the Academic Medical Center, the slides, blocks, and swabs were sent to DDL Diagnostic Laboratory for the second examination (by J. L.) 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 was used for high-grade AIN diagnosis (by hematoxylin-eosin staining), 2 sets of 3 × 4–µm sections were used for PCR of whole-tissue sections, 2 × 4–µm sections were evaluated by LCM (collected on PEN membrane slides from Carl Zeiss, Sliedrecht, the Netherlands), 2 × 4–µm sections were analyzed by p16 immunohistochemistry analysis, and a 4-µm section was evaluated for pathologic confirmation (by hematoxylin-eosin staining). After sectioning each block, the microtome was cleaned, and a new knife was used for the next block. The hematoxylin-eosin–stained PEN membrane slides were used for LCM PCR when multiple HPV types were found by PCR of whole-tissue sections. Negative controls (paraffin blocks) were used after every 10 blocks sectioned to check for cross-contamination.

**LCM Analysis**

When multiple HPV genotypes were detected by PCR of whole-tissue sections, LCM was used to obtain discrete areas of dysplastic anal epithelium from high-grade AIN lesions. As a control, 4 biopsy specimens with a single HPV type were analyzed. Slides were scanned using digital microscopy (Aperio Technologies, Vista, CA). An expert pathologist (J. L.) selected areas of AIN for sampling. A minimum of 1 LCM sample was collected from each high-grade AIN 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 the Zeiss P.A.L.M. microbeam UV LCM system and transferred to an AdhesiveCap500 opaque tube (Carl Zeiss). In addition, LCM was performed on a negative control block (composed of human placenta) for each case examined. DNA isolation and PCR for HPV DNA were performed as described below. Examples of LCM sections are 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 hours. Proteinase K was heat inactivated by incubation at 95°C for 10 minutes. Each DNA isolation run contained HPV-positive and HPV-negative controls.

The DNA isolation in swabs was performed using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), including pretreatment with proteinase K, according to the manufacturer’s instructions.

HPV DNA Detection and Genotyping
A 10-µL volume of isolated DNA was added to 40 µL of PCR mix. The short PCR fragment (SPF10) 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 SPF10 (version 1) DNA enzyme immunoassay [20]. Amplimers testing positive by DNA enzyme immunoassay were used to identify the HPV genotype by reverse hybridization with the HPV line probe assay (LiPA25), containing probes for the 25 most common mucosal HPV genotypes (ie, HPV6, 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]) [21].

PCR products were also tested with an in-house extended RHA strip, additionally detecting the less common mucosal HPV types 26, 30, 55, 61, 62, 64, 67, 69, 71, 82–85, 87, and 89–91, as described previously [22]. HPV55 and HPV64 are now classified as subtypes of HPV44 and HPV34, respectively [23].

RESULTS
Participants and Biopsy Specimens
We studied 31 high-grade AIN lesions obtained from 21 HIV-positive men who have sex with men. The median age of the participants was 48 years (interquartile range [IQR], 41–60 years), and they were known to be HIV positive for a median of 11 years (IQR, 5–16 years). The median CD4+ T-cell count was 480 cells/µL (IQR, 350–665 cells/µL), and 95% were using antiretroviral therapy. For patients with multiple biopsies, the biopsy specimens were taken from distinct macroscopic lesions.

Figure 1. Selection of dysplastic regions for laser-capture microdissection (LCM). A, Hematoxylin-eosin–stained biopsy. B, Multiple p16-positive dysplastic regions. C, Hematoxylin-stained slide with dysplastic regions selected for LCM: the 2 p16-positive regions contained human papillomavirus type 53 (HPV53) and HPV31. The p16-negative region did not contain HPV DNA.

Figure 2. Flowchart with single versus multiple human papillomavirus (HPV) types in the 31 high-grade anal intraepithelial neoplasia (HGAIN) biopsy specimens. WTS/PCR, polymerase chain reaction and HPV genotyping on whole-tissue sections. LCM/PCR, PCR and HPV genotyping on laser-capture microdissection–selected dysplastic regions.
### Table 1. Characteristics of 31 Anal Biopsies From 21 Human Immunodeficiency Virus (HIV)–Positive Men, With HPV Genotyping Results of Anal Swabs, Whole-Tissue Sections (WTS), and Laser-Capture Microdissection (LCM)–Selected Regions

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<th>WTS HPV Types</th>
<th>LCM Region AIN Grade</th>
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<th>Lesional HPV&lt;sup&gt;a&lt;/sup&gt;</th>
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LCM was performed in the 16 WTS containing multiple HPV types and in 4 WTS containing 1 HPV type (controls). A high-grade AIN biopsy occasionally contained both high-grade AIN and low-grade AIN regions.

Abbreviation: AIN, anal intraepithelial neoplasia.

<sup>a</sup> The combined HPV results of WTS and LCM.

<sup>b</sup> Collision lesions.

<sup>c</sup> Swab taken on the same day as biopsy.

<sup>d</sup> One HPV type could not be determined.
Figure 3. Multiple human papillomavirus (HPV) types in 2 regions. H-stained membrane slides. Two collision regions containing 2 HPV genotypes. A, Low-grade anal intraepithelial neoplasia (AIN) with HPV16 and HPV26 (biopsy number B5). B, High-grade AIN with HPV11 and HPV31 (biopsy no. B8).

Figure 4. Whole-tissue section with a collision region. Biopsy number B5 (Table 1) with a collision lesion containing both human papillomavirus (HPV) type 16 and HPV26. Both types were also found in separate regions. HPV31 was found in another separate region.
One biopsy specimen was obtained from 12 patients, 2 were obtained from 8, and 3 were obtained from 1.

**Analysis of Whole-Tissue Sections**

HPV was detected in all 31 whole-tissue sections. HPV genotyping analysis of whole-tissue sections of the 31 biopsy specimens showed a single HPV genotype in 15 whole-tissue sections (48%) and multiple HPV types in 16 (52%), as shown in Figure 2. The median number of HPV types per whole-tissue section was 2 (range, 1–7 types). Twenty different HPV genotypes were found. The predominant HPV type in the whole-tissue sections was HPV16 (present in 16/31), followed by HPV31 (in 6/31), and HPV18 and HPV51 (both in 4/31). Further details are presented in Table 1.

**Findings of LCM Analysis**

In the 16 whole-tissue sections containing >1 HPV type, 53 dysplastic regions (including low-grade AIN regions) were selected by LCM and analyzed. The median number of captured dysplastic regions per whole-tissue section was 3 (range, 2–6 dysplastic regions). In 14 of these 16 whole-tissue sections, a single HPV type was found in each LCM-selected region: in 10, only 1 HPV type was found in all regions, and in 4, different regions of the lesions contained different HPV types. Two of the 16 whole-tissue sections contained 2 HPV types in a single LCM-selected region. These were HPV16 and HPV26 (biopsy specimen B5) and 11 and 31 (biopsy specimen B8; Table 1 and Figures 3 and 4).

In 15 of 16 whole-tissue sections, all HPV types found in the LCM regions were also detected by PCR. One biopsy specimen had a lesion-specific HPV type that was not detected by PCR. It was an HPV of undetermined type (X-type; biopsy B22).

As controls, we performed LCM on 4 whole-tissue sections containing only 1 HPV type (biopsy specimens B14, B16, B17, and B20). All LCM regions contained the same single HPV type as observed in the whole-tissue sections (Table 1).

Furthermore, in 11 whole-tissue sections, LCM was used to select 15 nondysplastic regions, of which 10 were HPV negative. Four nondysplastic regions showed the same HPV type as adjacent dysplastic regions (Figure 5, biopsy B31). One nondysplastic region contained a HPV type (HPV91) that was not present in the adjacent dysplastic regions.

**HPV Detected in Lesions: Combined Result of Whole-Tissue Section Analyses and LCM**

In summary, 15 whole-tissue sections contained a single HPV type, and 16 contained multiple HPV types. In 10 of the latter, LCM-selected lesional regions contained the same HPV type in all dysplastic regions of the whole-tissue sections. Therefore, 25 (15 + 10) of 31 AIN lesions were associated with a single HPV type. In addition, 4 whole-tissue sections showed >1 HPV type,
but all were restricted to a single region within the whole-tissue section, yielding 29 of 31 biopsy specimens (94%) in which a single HPV type was found either on its own in the whole-tissue section or in a separate component of the high-grade AIN lesion. The remaining 2 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 2 HPV types are therefore collision regions involving 2 separate single-genotype HPV infections.

In the whole-tissue sections, we found 20 different HPV types, whereas, in total, 17 different lesional HPV types were found. HPV16 was a lesional HPV type in 14 of 31 (45%), followed by HPV18, HPV31, and HPV58 (all 3 were present in 3 lesions; Table 1).

HPV Analysis of Anal Swabs
Anal swabs for HPV analysis were available from 15 patients. For 6 patients, the anal swab specimens were collected on the same day as the biopsy, and for 9, the swab specimen was collected ≤ 4 weeks after biopsy. In total, 28 different HPV genotypes were found. The median number of HPV types per swab was 4 (range, 1–13 genotypes). The predominant HPV type was HPV16 (present in 7/15 swabs), followed by HPV18, HPV39, HPV51, HPV52, and HPV70 (all in 4/15 swabs; Table 1).

Eight of the 15 anal swab specimens did not contain all of the lesional HPV types found in the whole-tissue sections or LCM-selected areas. For patients with anal swab specimens obtained on the same day, 3 of 6 swabs did not contain all lesional types.

DISCUSSION
In this study, we performed HPV genotyping on whole-tissue sections and LCM-selected regions of high-grade AIN lesions in HIV-positive men who have sex with men. We identified the single and presumably causative HPV types in all regions of high-grade AIN lesions, although 2 LCM PCR samples contained a collision region. We showed that analysis of whole-tissue sections is not sufficient to determine the causative HPV type if multiple HPV types are present and that anal swabs often do not contain the causative HPV type. Finally, we found that almost half of the lesions were not caused by the 2 oncogenic HPV types (HPV16 and HPV18) 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 swab specimens taken from HIV-positive men who have sex with men showed multiple HPV types [24, 25]. Our study shows similar data in a group with confirmed high-grade AIN, 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 believe that this influenced our findings, because the high-grade AIN lesions had not yet been treated between the time of biopsy and swab collection and because 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 predicting high-grade AIN or detecting the causative HPV type in HIV-positive men who have sex with men. Given the high number of (oncogenic) HPV types in the anal canal in HIV-positive men who have sex with men and the possible presence of a lot of so-called 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 technology involving SPF10, DNA enzyme immunoassay, and LiPA25 (version 1) to identify the HPV types present (high analytical sensitivity). In general, very low levels of HPV do not reflect a clinically meaningful infection (ie, 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. A test that is too sensitive will result in lower specificity and a low positive predictive value (ie, the diagnosis of more irrelevant infections), which could result in excess follow-up tests, referrals, and treatment. On the other hand, despite the use of this very sensitive technology, testing of anal swabs still had insufficient sensitivity to detect the causative HPV type.

HPV genotyping of whole-tissue sections of biopsy specimens has also been used to identify HPV causing a lesion. However, it has previously been shown that high-grade AIN biopsy specimens often contain >1 HPV type [11]. The question is whether all HPV types found in a whole-tissue section play a role in the dysplasia or whether 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 [14–16]. In the current study, we established that when multiple HPV types were observed in whole-tissue sections, a single HPV type was found in each component of the high-grade AIN lesion in 29 of the 31 cases. In addition, the 2 lesions with a double HPV infection in an LCM sample appeared to represent collision regions between 2 lesions, each infected with a single HPV type. This confirms that the so-called one lesion, one virus concept previously proven for VIN and CIN lesions in immunocompetent women [14–16] is also valid for AIN lesions in HIV-positive men who have sex with men.

In addition to the dysplastic areas, we found that 5 of 15 LCM-selected nondysplastic areas were HPV positive. Four of those showed the same HPV type as adjacent dysplastic areas in the same tissue section. In 1 nondysplastic area, we found
an HPV type that was not present in the dysplastic LCM areas in the same tissues section. From our experience analyzing cervical specimens, we also see HPV DNA in normal epithelium in a small proportion of cases [26]. We have evidence that this form of latency occurs in resolving HPV infections, and we would expect it not to be uncommon in HIV-positive men.

Both AIN and anal cancer are far more prevalent among HIV-positive men who have sex with men than CIN and cervical cancer are among immunocompetent women [1, 27], and high-grade AIN in HIV-positive men who have sex with men is more often caused by non-HPV16/18 types [12]. More information is needed about the oncogenic capacity of these HPV types. If the pattern of genotype distribution found here in high-grade AIN 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-positive patients contained multiple HPV types. In the same study, 26 of 52 contained a nonvaccine high-risk HPV type [13]. Two smaller studies show a comparable proportion of nonvaccine HPV types in anal cancers of HIV-positive patients [12, 28]. Future multivalent HPV vaccinations, like the nonavalent vaccine currently evaluated in clinical trials, might be more appropriate for preventive programs focused on anal cancer [29].

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

In conclusion, almost each morphologically distinct area of high-grade AIN can be attributed to a single HPV type, as previously seen in CIN and VIN. Use of PCR sensitive for HPV in whole-tissue sections and LCM-selected regions can identify the lesional HPV type. The prevalence of high-grade AIN is very high among HIV-positive men who have sex with men, and future studies should focus on the malignant potential of different HPV types to target AIN prevention and treatment more efficiently.

Notes

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