Presence or absence of significant HPVE4 Expression in High-grade anal intraepithelial neoplasia with p16/Ki-67 positivity indicates distinct patterns of neoplasia

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ABSTRACT

Progression of anal intraepithelial neoplasia (AIN) involves transition from productive to transforming human papillomavirus (HPV) infection. Grading aims to distinguish productive low-grade AIN from high-grade (HG)AIN with risk of cancer. We describe immunohistochemical patterns in AIN adding a novel marker for initiation of the productive phase of the HPV life cycle (panHPVE4) to those for cell cycle activity (Ki-67) and transforming activity of HPV E7 gene (p16).

We studied 67 anal biopsies for suspected anal neoplasia (17 normal, 15 AIN1, 20 AIN2, 15 AIN3) from 54 men who have sex with men (MSM) at New York Presbyterian Hospital, USA. Two pathologists generated consensus AIN and immuno-grades. Whole tissue and laser capture microdissection (LCM) samples from multiple HPV-infected biopsies were tested for HPV with SPF10-PCR-DEIA-LiPA25 version 1.

(Para)basal Ki-67 expression distinguished normal from AIN (≥ lower-third Ki-67) with sensitivity 0.92 and specificity 1.0. Ki-67 did not distinguish grades of AIN.

Null/patchy P16 versus diffuse ≥lower-third patterns discriminated HGAIN (sensitivity 1.0; specificity 0.84). There was marked heterogeneity in E4 expression within HGAIN. Most AIN2 (14/20) was E4 positive versus 0/15 AIN3 (sensitivity: 0.70; specificity 1.0).

HPV was detected in 63 (94%) biopsies, with 49 (77.8%) high-risk HPV positive. HPV16 was the most frequent (13%). Multiple HPV genotypes were found in 15 (24%) biopsies and LCM-PCR confirmed specific HPV types in E4 +/- AIN.

While Ki-67 discriminated AIN and p16 high-grade AIN, E4+/p16+ staining shows that most AIN2 is different from transformed AIN3 in showing both entry into productive HPV infection and transforming activity.

INTRODUCTION

Over the last few decades, the incidence of anal cancer and its precursor anal intraepithelial neoplasia (AIN) has increased among specific groups including men who have sex with men (MSM), immunocompromised patients and women with a history of cervical or vulvar cancer [1-3]. The etiology of anal cancer is comparable to that of cervical cancer, with high-risk (hr)HPV detected in over 90% of anal cancers [4, 5], but, unlike cervical cancer, HPV16 has been detected in 80-90% of invasive anal cancer [6, 7]. HPV18 is uncommon and HPV33 is the second most important type, being found in 6% of anal cancers. Early detection of disease might improve survival and screening of high-risk groups such as MSM and HIV+ patients is advocated [8]. Anal Pap smear screening has been implemented in several clinics [9].

Abnormal anal Pap smear results are followed-up by high-resolution anoscopy (HRA) and biopsy. High-grade anal intraepithelial neoplasia (HGAIN; ≥AIN 2) is treated by either infrared coagulation (IRC) or electrocautery ablation (ECA) of the lesion. The cure rates are similar: 67%-75% for HIV+ patients and 80%-85% for HIV- patients [10, 11].

The histopathological grading of HGAIN is poorly reproducible and includes a very heterogeneous group of lesions, some of which regress spontaneously and others that progress to cancer. The overall risk of progression to cancer after treatment for HGAIN is low: 1.97% at 3 years [12] and 3.2% at 5 years [13]. It is therefore important to distinguish lesions with transforming characteristics that are likely to progress to cancer from productive lesions that are likely to regress. Biomarkers are needed that can be used in routine pathology practice, scored objectively and show distinguishing expression patterns between transforming and productive lesions. Immunohistochemical staining with p16 and Ki-67 has been identified as a valuable marker in diagnosis of HGAIN [14, 15]. Although Ki-67 is used widely as a marker of cell proliferation, it in fact identifies all cells that are undergoing cell cycle progression, including those supporting genome amplification in low-grade disease caused by both high- and low-risk (lr)HPV. These cells are 'in cycle,' but not proliferative [16]. P16 is a marker of transforming potential of hrHPV E7 gene [17]. The novel HPV-encoded marker panE4 is a surrogate for initiation of the productive phase of the papillomavirus life cycle, and is expressed in productive HPV infection [18]. It is currently being evaluated as an IHC marker of self-limiting productive HPV infection [19] in CIN.

An important feature of AIN in MSM and in HIV infection is the presence of multiple HPV types and we have previously shown by laser capture microdissection PCR (LCM-PCR) that each HPV type produces a separate lesion, although these lesions can sometimes overlap [20]. We therefore performed LCM-PCR on biopsies with multiple hr HPV types.

The aim of this study was to investigate if staining for E4 in addition to Ki-67 and p16 could provide the basis for a simple classification of AIN and particularly identify subdivisions of HGAIN.

METHODS

Selection of materials

Consecutive biopsies with diagnoses of normal anal transitional zone (N=17), AIN grade 1 (AIN1) (N=17), AIN grade 2 (AIN2) (N=17) and AIN grade 3 (AIN3) (N=17) were selected from the surgical pathology files of the New York Presbyterian Hospital. Biopsies were taken from 54 different MSM, among whom 48 (89%) were HIV positive. Diagnoses were based on the consensus diagnosis of two pathologists who individually reviewed the hematoxylin-eosin (H&E) sections of each biopsy.

The paraffin embedded biopsies were sent to DDL Diagnostic Laboratory. Sectioning was done using a sandwich method to give one 4-µm-thick section for confirmation of diagnosis (H&E before); 2 sets of 3*8-µm-thick (24 mm) sections for whole-tissue section (WTS)-PCR analysis; 1 slide covered with a polyethylene naphthalate membrane (Zeiss Microimaging GmbH, Jena, Germany) for LCM; and finally, 3 4-µm-thick section for immunohistochemical staining. An expert pathologist reviewed H&E slides from this new sandwich and an adjudicated consensus diagnosis of the original and review diagnoses was used as the histological reference outcome.

Immunohistochemistry

4 µm thick formalin-fixed paraffin embedded (FFPE) sections were used for immunohistochemical staining with $p16^{INK4a}$, Ki-67 and panHPVE4 using heat induced epitope retrieval with citrate buffer (Dako) and a primary mouse mAb anti-$p16^{INK4a}$ clone E6H4 (Ventana Medical Systems Inc, Roche, Mannheim, Germany), Ki-67 mAb (Dako, Agilent Technologies Inc, Santa Clara, USA) and panHPVE4 mAb FH1.1 (Labo Biomedical Products (LBP), Rijswijk, The Netherlands). The panHPVE4 antibody has been found to be reactive against HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, and 70 [19]. Reactivity was visualized using the EnVision Detection System (Dako, Agilent Technologies Inc, Santa Clara, USA).

Scoring of immunohistochemistry

$p16^{INK4a}$ (p16) immunostaining was classified as (0) no, or patchy (1) p16 positivity, (2) diffuse p16 staining restricted to the lower third of the epithelium, (3) diffuse p16 positivity up to two third of the epithelium or (4) diffuse full-thickness staining. In the evaluation

of p16 scores in relation to HGAIN, diffuse p16 staining of the lower one third of the epithelium or above (grade 2, 3 and 4) was considered positive.

Ki-67 was scored as (1) (para)basal up to lower third of the epithelium, (2) staining up to two third of the epithelium or (3) full thickness staining. In the evaluation of Ki-67 scores in relation to HGAIN, staining in the upper two third of the epithelium (grades 2 and 3) was considered positive.

PanHPVE4 immunoreactivity was scored as (0) negative, (1) focal —restricted to groups of cells in the upper quarter of the epithelium, and (2) extensive—upper half of the epithelium or more. Any grade of E4 positivity (grade 1 and 2) in the highest grade lesion identified was considered E4 positive in the detection of productive lesions. E4 positivity at the edge of a high-grade lesion adjacent to a low-grade lesion or normal epithelium was considered negative.

All slides were reviewed by two expert pathologists together, resulting in a consensus diagnosis.

HPV genotyping and laser capture microdissection

DNA for WTS-PCR was isolated from the FFPE material using a proteinase K procedure. To each sample, 100 µl of proteinase K lysis buffer was added and samples were incubated at 56 °C for 16–24 h. Proteinase K was heat-inactivated by incubation at 96 °C for 10 min. Specimens were tested for HPV DNA using the analytically sensitive SPF10-PCR-DEIA-LiPA25 version 1 system. The SPF10 PCR primer set amplifies a small fragment of 65 bp from the L1 region of mucosal HPV genotypes [21]. Amplification products were detected using the HPV SPF10PCR (version 1) DNA enzyme immunoassay (DEIA) system. DEIA-positive SPF10 amplimers were used to identify the HPV genotype by reverse hybridization with the HPV line probe assay (LiPA25), containing probes for 25 different 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]; SPF10HPV LiPA25 version 1 (LBP, Rijswijk, The Netherlands).

LCM was performed on all specimens with multiple high-risk HPV genotypes in the WTS-PCR. Regions from all lesions and normal epithelium were selected by an expert pathologist using H&E and p16 stained slides. Slides were scanned using digital microscopy (Aperio Technologies Inc, Vista, CA, USA). The number of selected regions ranged from 4 to 23, with an average region size of 49657µm^2 (range 8173µm^2-200390µm^2). Selected regions were excised with the 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 all specimens containing sufficient stroma, two stromal regions were selected as negative controls at the beginning and end of the LCM procedure. All PCR was performed as in WTS-PCR.

Statistical analysis

Analyses were performed using IBM SPSS version 22.0 (SPSS Inc, Chicago, IL) for Windows. Results were presented as absolute numbers and percentages. Sensitivities and specificities were calculated. The McNemar test was performed for analyses of nominal variables and P-values ≤0.05 were considered statistically significant.

RESULTS

A total of 67 blocks were graded on H&E slides and scored for IHC markers: 17 showed normal mucosa from the anal transitional zone, 15 AIN1, 20 AIN2, 15 AIN3. One sample was not adequate for grading and was excluded from analyses.

Immunohistochemistry

Sections were scored for p16, Ki-67 and E4 in the area of the most severe lesion identified on the H&E slide. Figures 1-3 show examples of AIN1-3 lesions stained for p16, Ki-67 and E4.

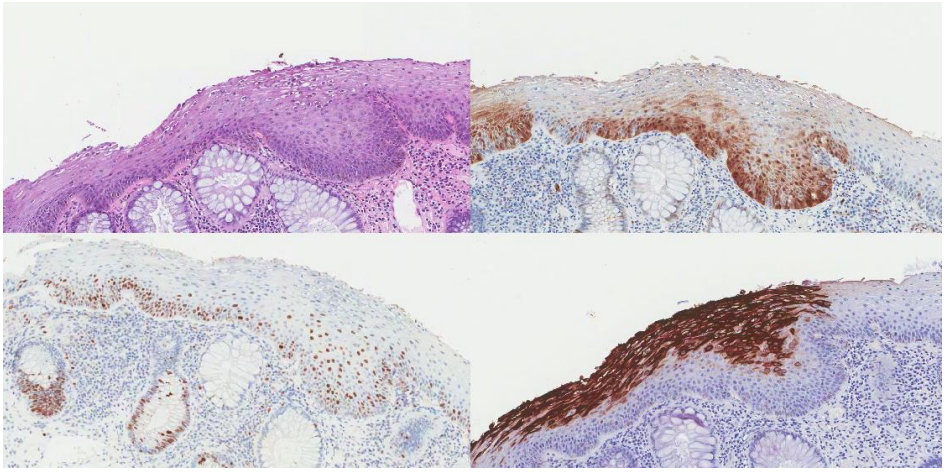

Figure 1. AIN1 lesion with patchy p16, Ki-67 staining in the lower 1/3 of the epithelium and extensive E4 staining. Note that the E4 and the Ki-67 staining overlap. This is cell cycle entry without cell division. The Ki-67 positive cells in the lower 1/3 of the epithelium are in cycle and some of these may be proliferating.

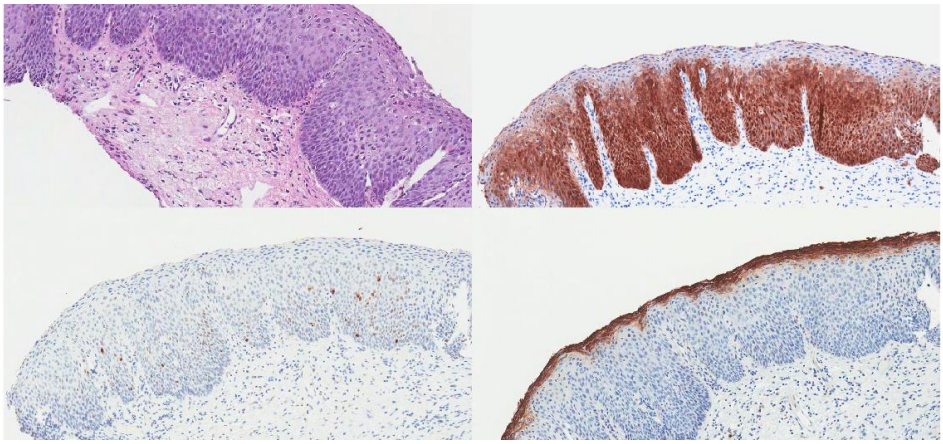

Figure 2. AIN2 lesion with HPV18 in the WTS, showing p16 staining in the lower 2/3 of the epithelium, Ki-67 staining in the lower 2/3 of the epithelium and extensive E4 staining of the superficial layers.

P16

P16 staining was negative or patchy in 27 lesions (40%), diffuse up to one third in 6 lesions (9%), up to two third in 14 (21%) and full thickness in 20 (30%). Biopsies of normal mucosa were completely negative or showed patchy staining for p16 in 16 (94%) specimens (Table 1). One biopsy (6%) graded on H&E as normal showed strong p16 staining in the lower 1/3 of the epithelium. In the AIN1 group, the majority of lesions (73%) showed patchy p16 staining, with the four lesions showing p16 positivity. All AIN2 lesions were p16 positive, with 85% showing p16 positivity above the lower 1/3 of the epithelium. In AIN3, p16 positivity was above 2/3 of the epithelium to full thickness in 60% of the cases. Of all HGAIN lesions, 89% showed p16 staining above the lower 1/3 of the epithelium.

Ki-67

Ki-67 was scored (para)basal to lower one third in 21 (31%), staining up to two third in 24 (36%) and full thickness in 22 (33%). Table 2 shows the Ki-67 positivity in biopsies from normal mucosa, AIN1, AIN2 and AIN3. Of all normal biopsies, 100% showed (para)basal Ki-67 staining or staining in the lower 1/3 of the epithelium. Most AIN (92%) showed Ki-67 positivity above the lower 1/3 of the epithelium. AIN1 showed different patterns of Ki-67 positivity, but in AIN2 staining mostly (65%) extended between the lower 1/3 and 2/3 of the epithelium, whereas the majority (67%) of AIN3 showed staining above the lower 2/3 of the epithelium to full thickness. Of the HGAIN lesions, 92% showed Ki-67 staining above the lower 1/3 of the epithelium.

E4

None of the normal biopsies were E4 positive. E4 positivity was very frequent in AIN1 (53%) and AIN2 lesions (70%). AIN1 lesions showed both extensive E4 positivity in 40% and focal positivity in 13%. A total of 9 (45%) AIN2 lesions showed extensive E4 positivity and focal positivity was seen in 5 lesions (25%). The division of focal or extensive positivity of E4 did not differ significantly between AIN1 and AIN2 (p=0.604).

Of the 15 AIN3 lesions, none showed E4 positivity in the AIN3 lesion (Table 3). Three AIN3 lesions showed E4 positivity located at the edges of the lesion (Figure 3). This should not be interpreted as representing E4 positivity in AIN3.

There was no significant difference in E4 positivity between cases with multiple and single HPV infections, HPV16 positivity and other HPV positivity or HIV positive and HIV negative patients.

Table 1 P16 scores in different grades of AIN.

	P16 score				
	Negative	*Patchy*	*<Lower 1/3*	*Lower 1/3-Lower 2/3*	*>Lower 2/3*
Normal (N=17)	8 (47%)	8 (47%)	1 (6%)	0 (0%)	0 (0%)
AIN1 (N=15)	0 (0%)	11 (73%)	1 (7%)	2 (13%)	1 (7%)
AIN2 (N=20)	0 (0%)	0 (0%)	3 (15%)	7 (35%)	10 (50%)
AIN3 (N=15)	0 (0%)	0 (0%)	1 (7%)	5 (33%)	9 (60.0%)

Table 2 Ki-67 scores in different grades of AIN.

	Ki-67 score		
Consensus diagnosis	*<Lower 1/3*	*Lower 1/3-Lower 2/3*	*>Lower 2/3*
Normal (N=17)	17 (100%)	0 (0%)	0 (0%)
AIN1 (N=15)	1 (6%)	7 (47%)	7 (47%)
AIN2 (N=20)	2 (10%)	13 (65%)	5 (25%)
AIN3 (N=15)	1 (6%)	4 (27%)	10 (67%)

Table 3 E4 positivity (negative, focal or extensive) in different grades of AIN.

	E4 score		
Consensus diagnosis	*Negative*	*Focal positivity*	*Extensive positivity*
Normal(N=17)	17 (100%)	0 (0%)	0 (0%)
AIN1 (N=15)	7 (47%)	2 (13%)	6 (40%)
AIN2 (N=20)	6 (30%)	5 (25%)	9 (45%)
AIN3 (N=15)	15 (100%)	0 (0%)	0 (0%)

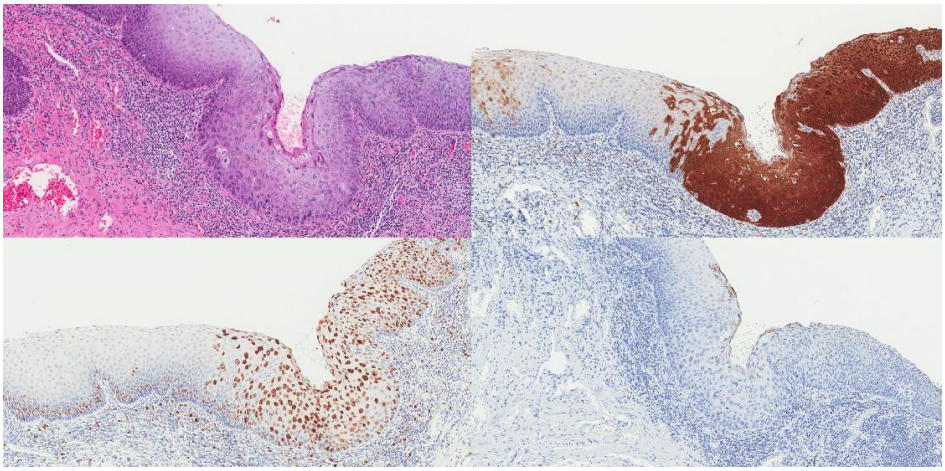

Figure 3. AIN3 lesion with HPV35 in the WTS, showing full thickness p16 positivity, full thickness Ki-67 staining and focal E4 staining at the edge of the AIN3 lesion where it merges with low-grade AIN. This should not be interpreted as E4 positivity in the AIN3 lesion.

HPV DNA

HPV DNA status of biopsies

With the analytically sensitive SPF10-PCR-DEIA-LiPA version 1, 63 (94%) of the biopsies were HPV positive, out of which 49 (77.8%) were hrHPV positive. Only 4/17 (24%) normal whole tissue sections were HPV negative, and these were also E4 and p16 negative. There were 48/63 (76%) HPV-positive samples with single HPV types detected. hrHPV types 16, 18, 31 or 33 were found in 19 (30%) of the HPV positive biopsies, with HPV16 found the most frequently (13%). Multiple HPV genotypes were found in 15 (24%) of HPV-positive biopsies, of which 7 contained multiple hrHPV genotypes. In 8 specimens a single hrHPV type was found in combination with lrHPV in WTS-PCR although multiple lrHPVs were also found and in 7 specimens there were multiple hrHPVs present. The genotype distribution based on these biopsies with a single type found by WTS-PCR and based on LCM-PCR of the worst lesions in a biopsy with a multiple infection with multiple hrHPV is shown in Table 4.

Relation between AIN grade and HPV type in cases with multiple hrHPV types selected for LCM-PCR

AIN grade and HPV type in multiple hrHPV infections by LCM-PCR

Seven WTS with multiple high-risk HPV genotypes were selected for LCM-PCR. One WTS had a highest lesion grade AIN3, five had AIN2 and one was normal. Four had 2 genotypes found in the WTS (58%), one had 3 genotypes (14%), one had 4 genotypes (14%) and one had 5 genotypes (14%). Four samples contained a multiple infection with HPV16 DNA and other genotypes.

In four lesions, not all genotypes were found with LCM-PCR (57%). In one lesion (14%), 2 additional HPV genotypes were detected with LCM-PCR and in the remaining 3 lesions (42%), all genotypes found in the WTS.

Figure 4 shows the distribution patterns of immunohistochemical staining of an AIN2 lesion for the three markers together with annotated regions selected for LCM-PCR. On WTS-PCR, genotypes 16, 31, 51, 52 and 70 were detected. Both p16 and Ki-67 showed full thickness staining and the E4 showed extensive positivity. With LCM-PCR, HPV51 was found in the p16 positive, Ki-67 positive and E4 positive AIN2 region and was designated as the causative type of the lesion. HPV 16, 31, 52 and 70 were all detected in regions of adjacent AIN1 lesions. HPV 18 was found by LCM-PCR in two small regions although not detected by WTS-PCR in the presence of multiple other types.

Table 4. Frequencies of single HPV genotypes detected in WTS-PCR and LCM-PCR of 51 biopsies with different grades of AIN by worst grade of AIN in the biopsy. N=3 genotypes unknown.

Consensus diagnosis	HPV16	HPV18	HPV31	HPV33	HPV35	HPV39	HPV45	HPV51	HPV52	HPV56	HPV58
Normal	2	1	0	0	1	0	1	0	0	0	0
AIN1	1	0	1	0	0	1	0	0	0	0	0
AIN2	2	3	1	2	0	2	1	2	1	2	0
AIN3	3	0	1	2	1	0	0	0	2	0	0
Consensus diagnosis	**HPV59**	**HPV66**	**HPV68**	***HPV6***	***HPV11***	***HPV40***	***HPV44***	***HPV53***	***HPV54***	***HPV70***	***HPV74***
Normal	0	0	0	0	2	0	0	0	0	0	0
AIN1	0	0	1	4	2	1	1	0	0	0	0
AIN2	1	0	0	0	0	0	0	2	0	0	0
AIN3	0	1	0	0	0	0	0	0	0	0	0

Classification of AIN lesions by patterns of E4, p16 and Ki-67 expression in relation to HGAIN grade and associated hrHPV

Patterns of positivity for the IHC markers E4, p16 and Ki-67 in 67 specimens are shown in Table 5. In this analysis the cut-off used for each marker was for E4 any positivity (E4≥1) as a marker of productive activity; and for p16 positivity involving 1/3 or more (p16≥2) of the epithelium as a marker of transformation. For Ki-67 abnormal expression was defined as Ki-67 positivity above the lower 1/3 of the epithelium (Ki-67≥2). HPV genotype association was categorized as low-risk HPV (LR), when only low-risk HPV is found, high-risk HPV (HR) when at least one high-risk HPV genotype was found, or HPV negative.

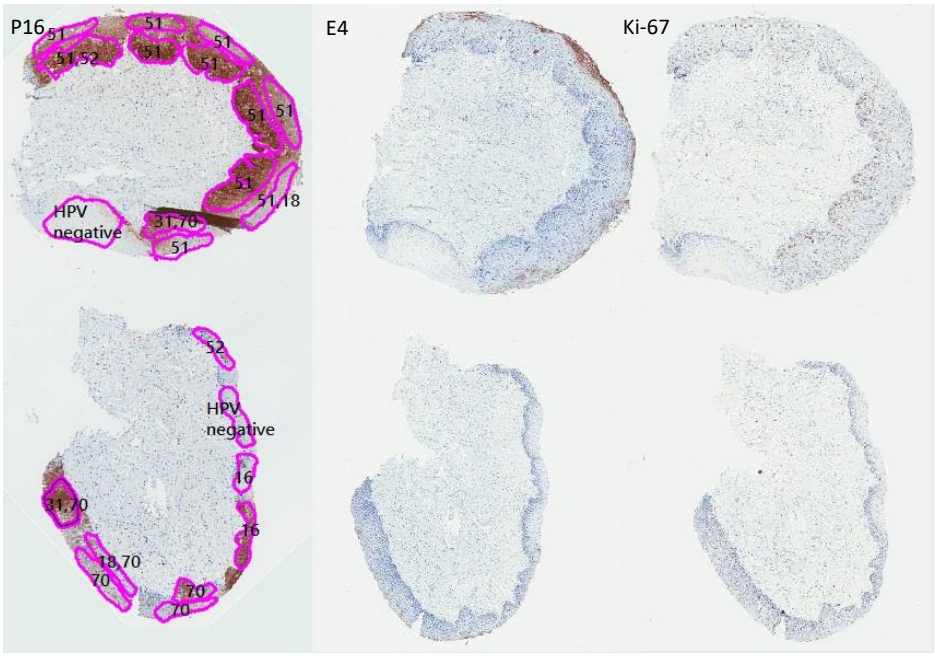

Figure 4. P16 (full thickness staining), E4 (extensive staining) and Ki-67 (full thickness staining) slides with the worst diagnosis of AIN2, with HPV genotypes 16, 31, 51, 52 and 70 detected in the whole tissue section SPF10-PCR-DEIA-LiPA25. LCM-PCR analysis assigned all genotypes to different lesions in the biopsy, and in addition, detected HPV 18 concentrated in two discrete regions.

Normal epithelium showed no positivity for E4 or p16 by the criteria defined above and only Ki-67 expression confined to the lower 1/3, although both lrHPV and hrHPV DNA could be detected in this group. In AIN1 14/15 (93%) lesions were Ki-67 positive, but only 3/15 (20%) were p16 positive (all hrHPV) and 6 with hrHPV were p16 negative. E4 was expressed in 8/15 (53%) AIN1 cases of which 6 were lrHPV and 2 were hrHPV and 7/8 (87%) E4 expressing AIN1 were p16 negative.

In AIN2, all were p16 positive of which 2 were Ki-67 negative. All were hrHPV positive. E4 positivity was seen in 12/17 (70%) p16 positive cases and in 2/3 p16 negative cases. E4 positivity was not seen in AIN3; none were E4 positive. However, all AIN3 cases were hrHPV positive and p16 positive.

Almost all AIN1, AIN2 and AIN3 lesions showed extensive Ki-67 positivity above the lower 1/3 of the epithelium. There is a clear relation between p16 expression and presence of HGAIN, with 100% of HGAIN being positive compared to 4/15 (27%) AIN1.

Table 5. The expression of E4, p16 and Ki-67 in different grades of AIN, identifying productive lesions (E4≥1) and transforming lesions (p16≥2) and proliferative activity (Ki-67≥2) and the detection of low-risk (LR) and high-risk (HR) HPV in the WTS.

	Early HPV infection or non-HPV-associated pathology		Mature productive HPV infection	Productive early transforming HPV infection		Transforming HPV infection	
Diagnosis	**E4-/Ki-67-/ p16-**	**E4-/Ki-67+/ p16-**	**E4+/Ki-67+/p16-**	**E4+/Ki-67-/ p16+**	**E4+/Ki-67+/p16+**	**E4-/Ki-67-/ p16+**	**E4-/Ki-67+/ p16+**
Normal	16	0	0	0	0	1	0
HPV	*HR: 8*					*HR: 1*	
	LR: 4					*LR: 0*	
	HPV negative: 4					*HPV negative: 0*	
AIN1	0	5	6	1	1	0	2
HPV		*HR: 4*	*HR: 0*	*HR: 1*	*HR: 1*		*HR: 2*
		LR: 1	*LR: 6*	*LR: 0*	*LR: 0*		*LR: 0*
		HPV negative: 0	*HPV negative: 0*	*HPV negative: 0*	*HPV negative: 0*		*HPV negative: 0*
AIN2	0	0	0	0	14	2	4
HPV					*HR: 14*	*HR: 2*	*HR: 4*
					LR: 0	*LR: 0*	*LR: 0*
					HPV negative: 0	*HPV negative: 0*	*HPV negative: 0*
AIN3	0	0	0	0	0	1	14
HPV						*HR: 1*	*HR: 14*
						LR: 0	*LR: 0*
						HPV negative: 0	*HPV negative: 0*

There is, however, an important difference in expression of E4 within HGAIN, between AIN2 and AIN3. Most, 12/17 AIN2 (70%) cases were E4 positive whereas all AIN3 cases were E4 negative. The presence of E4 associated with strong p16 positivity in HGAIN lesions indicates that HGAIN includes cases (14/35; 40%) where there is evidence of continuing productive phase of the HPV life cycle associated with productive infection alongside evidence of transforming activity. This pattern of E4 and p16 expression is seen in AIN2.

DISCUSSION

This is the first comprehensive study showing that a panel of immunohistochemical markers consisting of E4, P16 and Ki-67 makes it possible to differentiate between

normal anal mucosa and discrete lesions of AIN1, AIN2, and AIN3 in a representative population of MSM with a high frequency of HIV infection. Our results indicate that Ki-67 is a useful marker discriminating AIN from normal, p16 is useful in discriminating high-grade AIN from normal/low-grade AIN, and E4 is useful in discriminating AIN2 from AIN3 and shows that AIN2 is not a homogeneous group.

Between normal and AIN1, AIN2 and AIN3 there is a change in expression of markers indicating productive HPV infection with initiation of the productive phase of the HPV life cycle (E4), cell cycle entry (Ki-67) and potential transforming activity of the HPV E7 gene (p16). Importantly it shows that HGAIN (AIN2 and AIN3) which is the current threshold for treatment is far from homogeneous with regard to the balance between HPV productive activity and transformation, reflected partially in histological grading into AIN2 and AIN3.

Ki-67 staining extended above the lower third in most AIN of all grades. Its expression is a consequence of entry into an incomplete cell cycle for viral genome amplification in productive infections associated with both lrHPV and hrHPV, as well as an increase in cell proliferation in transforming hrHPV infection [22]. Expression of p16 increases with grade of AIN and is negative or patchy in all normal biopsies. AIN1 showed usually only patchy p16 staining but almost 1/3 lesions showed diffuse staining of at least the lower third. In HGAIN there was extensive p16 staining above the lower 1/3 of the epithelium in 89% lesions, compared to 19% AIN1. E4 was negative in all normal biopsies. Many (56%) AIN1 lesions were E4 positive. Importantly we found that in HGAIN, although all were p16 positive and also Ki-67 positive there was marked heterogeneity in E4 expression. AIN2 lesions showed E4 positivity in 70% of the cases, within which most showed extensive E4 positivity. In AIN3 lesions, no E4 positivity was seen in the lesion. These results indicate that there is an important difference in the balance between initiation of the productive phase of the HPV life cycle and transforming activity between AIN2 and AIN3. This switch may be linked to increasing HPV genomic integration.

Previous biomarker data based on p16 with or without the addition of Ki-67 [23-26] supported a 2-tiered system separating low-grade lesions and high-grade lesions, with recommendations for treatment of all HGAIN, rather than a 3-tiered system separating AIN1-3 and CIN1-3 [27]). There is, however, other evidence that intraepithelial neoplasia grade 2 (–IN2) represents a mixture of regressive and progressive disease. The present data supports the view that a division into LGAIN and HGAIN may be too simplistic, and needs further investigation in relation to the known infrequency of progression of HGAIN to invasive cancer.

The marker patterns do not precisely conform to the conventional histological grades. These findings indicate 4 distinct patterns of lesion development with likely progression from HPV infection to productive and increasingly transforming HPV-induced lesions. Lesions called AIN1 include early or resolving HPV lesions or non-HPV-associated pathology, even if HPV DNA is being carried (E4 negative, Ki-67 positive, p16 negative), with a proportion of AIN1 being mature productive HPV lesions (E4 positive, Ki-67 positive, p16 negative) completing the HPV life cycle. AIN2 is predominantly composed of lesions that show both productive and transforming features (E4 positive, Ki-67 positive, p16 positive) and AIN3 is predominantly a transforming lesion (E4 negative, Ki-67 positive, p16 positive). The presence of two patterns of Ki-67 and p16 expression with or without E4 expression is consistent with the suggestion that –IN2 includes both progressive and regressive lesions. However, the presence of E4 expression, indicating that some HPV infected basal cells in a lesion have matured and complete the HPV life cycle, does not mean that all basal cells are not transforming. Transformation of basal cells might occur concurrently or sequentially following the progress of E4 positive cells up the epithelial differentiation pathway. It is also possible that as proposed for the cervix, different zones of anal epithelium, particularly immature cells in the transformation zone, may be more susceptible to transforming than productive hrHPV infection [28]. However, full transformation is not a single stage process and the continued expression of some E4 is likely to indicate an early stage in the process.

This hypothesis needs further exploration in progression studies of AIN and of CIN using E4 and other markers such as methylation of tumor suppressor genes and expression of other cellular transforming genes in relation to progression and regression.

Attention to the distribution of E4 is important. E4 expression can be a result of productive low risk HPV infections [29 30]. Therefore, interpretation of E4 staining patterns in multiple infections needs to be done carefully. Focal E4 expression may also be seen at the edges of HGAIN lesions (Figure 3), where the more normal, local epithelial microenvironment is different from that within the lesion and may promote epithelial differentiation and entry into the productive phase of the HPV life cycle rather than representing the true nature of the lesion as a whole. It is also important to distinguish E4 expression in the lesion from E4 expression in collision areas between a HSIL caused by hrHPV and an adjacent AIN1 expressing E4 in cases of multiple infection. This AIN1 lesion might develop from basal cells that follow a different pathway leading to a productive infection and such independent event does not represent a precursor of the adjacent AIN3.

We confirmed the importance of understanding the role of multiple genotypes by laser capture microdissection analysis of whole tissue sections that contained multiple hrHPV

genotypes to assign one HPV genotype to the worst lesion according to the one virus one lesion theory [20, 31]. In the case of multiple infection in the whole tissue section, single HPV genotypes can be found in different regions of the specimen. It was previously shown in cervical specimens that HPV DNA can also be found in normal epithelium and these genotypes can be considered passengers or latent infections [31].

We did not analyze all non-dysplastic regions using LCM-PCR. Most lesional areas contained only a single HPV type, but multiple genotypes were found in 10/97 LCM regions. These all appeared to be collision of 2 different lesions infected with different genotypes.

AIN1 lesions that do not express p16 and/or E4 need to be explored further, particularly as similar lesions in the cervix are poorly reproducible in diagnosis by pathologists [19], and may represent developing or regressing productive HPV-induced lesions or lesions due to other causes in which HPV DNA is found coincidentally. E4 is a marker of completion of the life cycle and therefore is not necessarily expressed in very early developing CIN1 lesions, in resolving CIN1 or in latent infections. In the present descriptive study, we found that 5/15 (33%) of AIN1 lesions express no E4 and no diffuse p16 staining. These lesions might fall into a not-HPV-related category, or represent early or resolving HPV infections. Furthermore, 2/15 AIN1 lesions (13%) express no E4 but did show diffuse p16 staining, suggesting that these lesions are more like AIN2.

The findings in this study of AIN are similar to those in previous studies of CIN. Van Baars et al. found E4 positivity in 21.5% of the CIN3 lesions, 47.4% of the CIN2 lesions, 58.8% of the CIN1 lesions and 0% of the negative biopsies [32]. The proportions of E4 positivity in CIN correspond generally to the E4 positivity found in AIN lesions. The E4 positivity that is found in AIN2 lesions (70%) however is considerably higher than in CIN2. Even though the HPV genotypes and marker patterns of AIN and CIN are comparable, there are important differences between HIV+ MSM in AIN and women in the reproductive age in CIN. In the present study, the group of HIV negative cases was very small (11%) and therefore did not allow comparative analyses between HIV+ and HIV- cases. Most studies on inter-observer agreement of immunohistochemical markers have been done on cervical specimens, with good to excellent interobserver agreement for immunohistochemical markers E4, p16 and proliferation marker MCM [32] in cervical biopsies.

Combining p16 and Ki-67 with E4 raises the possibility of distinguishing between productive and transforming AIN lesions. Studies are needed to explore further the clinical utility of E4/p16 biomarkers in identifying progressive HGAIN lesions requiring treatment. The relation of these markers to other molecular markers, such as methylation of tumor suppressor genes, with the ability to identify advanced transforming lesions as

proposed in CIN3 is important [33, 34]. Demonstration of effective differentiation between transforming and productive lesions could prevent current over-treatment of all HGAIN lesions, the majority of which do not progress and many of which may go into spontaneous regression.

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