Grading immunohistochemical markers p16INK4a and HPV E4 identifies productive and transforming lesions caused by low- and high-risk HPV within high-grade anal squamous intraepithelial lesions

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ABSTRACT

Aims: Current LAST guidelines recognise high- and low-grade anal squamous intraepithelial lesions (HSIL, LSIL) and recommend treatment of all HSIL, but not all HSIL progress to cancer. This study aims to distinguish transforming and productive HSIL by grading immunohistochemical (IHC) biomarkers p16 and E4 in lr- and hr-HPV- associated SIL as a potential basis for more selective treatment.

Methods and results: Immunostaining for p16 and HPV E4 was performed and graded in 183 biopsies from 108 HIV+ MSM. Causative HPV genotype of the worst lesion was identified using SPF10-PCR-DEIA-LiPA25v1, with laser capture microdissection (LCM) for multiple infections. Worst lesions were scored for p16 (0-4) to identify activity of hrHPV E7 gene, and panHPV E4 (0-2) marking HPV production and life-cycle completion. There were 37 normal, 60 LSIL and 86 HSIL with 85% LSIL caused by lrHPV and 93% HSIL by hrHPV. No normal biopsy showed E4 but 43% of LSIL and 37% of HSIL were E4 positive. No differences in E4 positivity rates were found between lrHPV and hrHPV lesions. Most (90%) lesions caused by lrHPV showed very extensive patchy p16 staining. p16 grade in HSIL was variable with frequency of productive HPV infection dropping with increasing p16 grade.

Conclusions: Combined p16/E4 IHC identifies productive and non-productive HSIL associated with hrHPV within the group of HSIL defined by the Lower Anogenital Squamous Terminology recommendations. This opens the possibility of investigating selective treatment of hrHPV-caused advanced transforming HSIL and a "wait and see" policy for productive HSIL.

INTRODUCTION

The incidence of squamous cell carcinoma of the anal canal and anal intraepithelial neoplasia (AIN), also called squamous intraepithelial lesions (SIL), is increasing, especially in high-risk populations such as men who have sex with men (MSM), HIV infected patients and women with a history of a vulvar or cervical HPV-related malignancy [1-3]. High-risk (hr)HPV is detected in over 80% of the anal cancers [4-8] and carcinoma associated with low-risk (lr)HPV is rare [9]. Because of the similarities in aetiology and pathology between cervical and anal intraepithelial neoplasia, the clinical approaches to these lesions are similar. In the case of a suspected anal high-grade SIL (HSIL), patients are subjected to a high-resolution anoscopy during which biopsies are taken of abnormal appearing regions, and treatment follows after confirming diagnosis of HSIL by histopathology, which has low inter and intra observer agreement [10, 11].

The Lower Anogenital Squamous Terminology (LAST) recommendations recognise only low and high-grade SIL [12]. This separation is based on the assumption that LSIL represents a productive HPV infection that will regress whereas HSIL is considered to be a transforming HPV infection that has a high chance of progression to cancer and is in need of treatment. However, it is estimated that only 10% of anal HSIL ultimately progress to cancer [7] if left untreated and about 47% of shows regression [13, 14]. The LAST recommendations for pathological diagnosis make only limited use of immunohistochemical biomarkers. The LAST recommendations state that diagnosis of HSIL should be made using H/E histopathology supported by the use of p16 as a surrogate marker for hrHPV E7 transforming gene activity, only to confirm HSIL diagnosis in case of uncertainty or disagreement about LSIL versus HSIL to show presence of diffuse p16 positivity [12].

A biomarker specific for productive HPV infection such as HPV E4, in combination with patterns of diffuse p16 expression as a marker of the transforming activity of the hrHPV E7 gene, might help to classify more objectively anal intraepithelial lesions (AIN), both LSIL and HSIL, and provide a basis for more selective treatment, avoiding unnecessary intervention for self-limiting lesions. Currently the LAST recommendations recommend all HSIL is treated. There are several treatment modalities for anal HSIL including infrared coagulation, electrocautery, surgical excision and topical application of trichloroacetid acid or imiquimod. Currently, electrocautery is the treatment of choice for intra-anal HSIL in many centers [15]. However, there is no international consensus guideline, recurrence rates are high for all modalities and all can cause side effects like pain and anal blood loss [16]. Selective treatment of only men HSIL with a higher chance of progression to cancer could prevent overtreatment with negative side effects.

E4 is a marker of completion of the HPV life cycle seen in productive HPV infection associated with low-grade CIN or AIN [17, 18]. Expression of E4 in HPV infected differentiated squamous cells results in disruption of the keratin filamentous network of the squamous cell, inhibits formation of the cornified envelope, and plays a role in virus release and transmission [19].

p16 is diffusely overexpressed in high-grade intraepithelial lesions and carcinomas driven by hrHPV, and is induced by HPV E7 of high-risk HPV types [12, 20, 21]. It is a protein regulating the G1 to S phase checkpoint of the cell cycle.

Previously we have shown that AIN2 differs from AIN3 in its expression of abundant E4 and no HPV E4 was found in AIN3 lesions [22]. In this study we demonstrate the heterogeneity of HSIL lesions using a combination of IHC biomarkers p16 and E4 within the category of HSIL defined by current practice using LAST recommendations [12]. We determined the causative HPV genotype of SIL and related the HPV genotype to the p16 and HPV E4 biomarker expression pattern to show that based on patterns of biomarker expression of p16/E4, we can identify productive HSIL associated with hrHPV and SIL associated with lrHPV. This provides a potential basis for selection of cases currently treated as HSIL that could be appropriate for a "wait and see" policy and not require immediate treatment.

MATERIALS AND METHODS

Study population

For the present study, 183 biopsies from two studies were used:

Biopsies from the H2M2 cohort study were selected[23].

H2M2 is a multicentre prospective cohort study of HIV-infected MSM aged ≥18, conducted at several clinics in Amsterdam. Men had anal HPV testing every 6 months for 2 years and in the course of regular care they were offered anal screening using high-resolution anoscopy (HRA). At the initial HRA, biopsies were taken from HSIL suspected areas, detecting HSIL in at least 1 biopsy in 50 men. All other biopsies from these 50 men were also included, resulting in a total number of 116 biopsies.

A second group was selected from the pathology files of the New York Presbyterian Hospital . This group consisted of 67 biopsies from 53 MSM of whom 47 were HIV+. 60/67 were from HIV+ MSM. Biopsies from this second group had been previously stained with

biomarkers p16, Ki-67 and E4 for the description of expression patterns in different grades of AIN [22].

Histology processing and review

The formalin-fixed paraffin-embedded (FFPE) material of all included biopsies was cut at DDL, Rijswijk Subsequent slides were used for: 4µm slides for Haematoxylin and Eosin (H/E) staining (before and after), 3 x 4µm slides for immunohistochemical staining (p16 and E4), 1 membrane slide for laser capture microdissection (LCM) and 1 tube (3*8µm) for HPV detection.

Two specialized pathologists reviewed the H/E slide first individually and then together and made a diagnosis which was used as our consensus diagnosis. Then, the p16 slide was used to confirm detection of HSIL in a set of AIN1 and all AIN2, in an approach based on the LAST recommendations:

- Histologically normal: normal.
- Histologically AIN1 (no suspicion of AIN2): LSIL.
- Histologically AIN1 (suspicion of AIN2 by at least one pathologist) or AIN2, p16 negative: LSIL.
- Histologically AIN1 (suspicion of AIN2 by at least one pathologist) or AIN2, p16 diffusely positive: HSIL.
- Consensus diagnosis AIN3: HSIL.

In further analyses, the consensus diagnosis and the LAST diagnosis were used for comparison.

HPV genotyping and LCM

HPV genotyping of whole tissue sections was done using the analytically sensitive SPF10-PCR-DEIA-LiPA25 version 1, genotyping 25 lr- and hr-HPV types [24]. The causative genotype of the highest graded lesion present on biopsy was attributed to the genotype found in the whole tissue section (WTS) in case of a single infection in ≥AIN1 biopsies. From biopsies in which multiple HPV genotypes were found, the worst lesions was selected for laser capture microdissection to identify the causative type of this worst lesion. Laser captured worst lesions were analysed according to the same HPV testing algorithm (SPF10) [25].

Immunohistochemistry

4 µm thick FFPE sections were used for immunohistochemical staining with $p16^{INK4a}$ and panHPVE4 using heat induced epitope retrieval with citrate buffer (Dako) and a primary mouse monoclonal antibody anti-p16INK4a clone E6H4 (Ventana Medical Systems Inc,

Roche, Mannheim, Germany), and XR-E4-1 (Labo Biomedical Products (LBP), Rijswijk, The Netherlands). The panHPVE4 antibody has been found to be reactive against at least HPV genotypes 6,11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, and 70[17, 22]. Reactivity was visualized using the EnVision Detection System (Dako, Agilent Technologies Inc, Santa Clara, USA).

All slides were scored jointly for p16 grade and E4 positivity by two expert pathologists, resulting in an immunoscore for each marker.

p16^{INK4a} immunohistochemistry

p16^{INK4a} (p16) immunostaining was classified as no staining (grade 0) or patchy p16 positivity (grade 1), diffuse p16 positivity restricted to the lower third of the epithelium (grade 2), diffuse p16 positivity restricted to the lower two third of the epithelium (grade 3) or diffuse staining above the lower two third up to full-thickness staining (grade 4). In the evaluation of p16 scores in relation to LAST classification of HSIL, diffuse p16 staining (grade 2, 3 and 4) was considered positive.

HPV E4 immunohistochemistry

Pan HPVE4 immunoreactivity in the worst lesion was scored as negative (grade 0), focal —restricted to groups of a few cells in the upper layers of the epithelium (score 1), and extensive—upper half of the epithelium or more (score 2) [17]. Any E4 positivity (score 1 and 2) in the highest-grade lesion identified was considered E4 positive. E4 positivity at the edge of a high-grade lesion adjacent to a low-grade lesion or normal epithelium was considered negative as in previous studies [22].

Statistical analyses

Results were analysed using IBM SPSS version 22.0 for Windows (Chicago, IL). Data were presented as absolute numbers and percentages. Percentages were compared using the Chi-square test and the level of statistical significance was set at $p<0.05$.

RESULTS

In 183 biopsies from 108 patients, classified by the LAST recommendations applied by two expert pathologists there were 37 normal, 60 LSIL, and 86 HSIL as the worst lesions seen in the biopsies. Expert H/E consensus diagnosis using the AIN classification was negative in 37 biopsies, AIN1 in 67 biopsies, AIN2 in 43 biopsies and AIN3 in 36 biopsies. Table 1 compares consensus AIN diagnoses with LAST diagnoses.

Immunohistochemical marker scoring

Results of p16 and E4 scoring of the worst areas of lesions in different grades of AIN and SIL are shown in Tables 2 and 3.

p16 score increased with the severity of the lesion, with 83% (86/104) of all negative/AIN1 lesions and 93% (90/97) negative/LSIL lesions by LAST criteria showing no or patchy p16 staining. Of all ≥AIN2 lesions, 89% (70/79) showed diffuse p16 staining above the lower 1/3 of the epithelium, with 58% of AIN2 showing diffuse staining above the lower 2/3 of the epithelium and 78% of AIN3 showing this pattern. Using the LAST diagnosis of HSIL, only 1% (1/86) showed patchy staining and 67% showed staining of >2/3 epithelium.

E4 was negative in 100% of the normal biopsies, while 49% (33/67) of the AIN1 lesions scored positive for E4. In AIN2, 56% (24/43) of the lesions were E4 positive and only one of the AIN3 lesions was E4 positive (3%, 1/36) ($p<0.001$). When using the LAST diagnosis, 26/60 (43%) of LSIL and 32/86 (37%) of HSIL were E4 positive ($p=0.457$).

Table 1: Consensus diagnosis based on H/E using AIN classification compared to p16-supported LAST diagnosis.

Consensus Diagnosis (%)	LAST diagnosis (%)		
	Normal (N=37)	LSIL (N=60)	HSIL (N=86)
Normal (N=37)	37 (100%)	0	0
AIN1 (N=67)	0	56 (84%)	11 (16%)
AIN2 (N=43)	0	4 (9%)	39 (91%)
AIN3 (N=36)	0	0	36 (100%)

Table 2: p16 scores in biopsies with different grades of AIN by the Consensus Diagnosis (2a) and the LAST diagnosis (2b).

2a. Consensus Diagnosis	p16 score				
	Negative (grade 0)	*Patchy (grade 1)*	*≤Lower 1/3 (grade 2)*	*≤Lower 2/3 (grade 3)*	*>Lower 2/3 (grade 4)*
Normal (N=37)	20 (54%)	16 (43%)	1 (3%)	0	0
AIN1 (N=67)	1 (2%)	49 (73%)	4 (6%)	7 (10%)	6 (9%)
AIN2 (N=43)	0	4 (9%)	3 (7%)	11 (26%)	25 (58%)
AIN3 (N=36)	0	1 (3%)	1 (3%)	6 (16%)	28 (78%)
2b. LAST Diagnosis	p16 score				
	Negative (grade 0)	*Patchy (grade 1)*	*≤Lower 1/3 (grade 2)*	*≤Lower 2/3 (grade 3)*	*>Lower 2/3 (grade 4)*
Normal (N=37)	20 (54.1%)	16 (43.2%)	1 (2.7%)	0	0
LSIL (N=60)	1 (2%)	53 (88%)	3 (5%)	2 (3%)	1 (2%)
HSIL (N=86)	0	1 (1%)	5 (6%)	22 (26%)	58 (67%)

Table 3: E4 scores (negative, focal or extensive) in biopsies with different grades of AIN by the consensus diagnosis (3a) and the LAST diagnosis (3b).

3a. Consensus Diagnosis	E4 score		
	Negative (grade 0)	*Focal positivity (grade 1)*	*Extensive positivity (grade 2)*
Normal (N=37)	37 (100%)	0	0
AIN1 (N=67)	34 (51%)	7 (10%)	26 (39%)
AIN2 (N=43)	19 (44%)	8 (19%)	16 (37%)
AIN3 (N=36)	35 (97%)	0	1 (3%)
3b. LAST Diagnosis	E4 score		
	Negative (grade 0)	*Focal positivity (grade 1)*	*Extensive positivity (grade 2)*
Normal (N=37)	37 (100%)	0	0
LSIL (N=60)	34 (57%)	5 (8%)	21 (35%)
HSIL (N=86)	54 (63%)	10 (12%)	22 (25%)

HPV genotyping of the worst lesion

Single HPV infections

Whole tissue sections (n=183) were tested for HPV positivity and genotyped, resulting in 9 HPV negative biopsies, 120 with a single HPV genotype, of which 11 could not be genotyped (type X), and 54 with multiple genotypes present. The most frequently found HPV genotype as a single infection was HPV6 (22 biopsies from 18 patients), followed by HPV16 (17 from 16 patients).

The causative genotype of the worst lesion present on biopsy was attributed to the genotype found in the WTS in case of a single infection in ≥LSIL biopsies. Biopsies in which no abnormal epithelium was found were excluded when identifying the causative type (19/120 single infections).

Multiple HPV infections

The causative type of the worst lesion in the 45/54 biopsies with multiple infections was identified using LCM: nine biopsies contained normal anal epithelium only and were not further analysed. The worst diagnosis of the remaining 45 biopsies was LSIL in 10 and HSIL in 35.

In total, a causative genotype was identified for 139/146 worst lesions and seven worst lesions were HPV positive but could not be genotyped (type X). Supplementary table 1 shows the causative genotype in LSIL and HSIL according to LAST diagnosis. Most LSIL was caused by lrHPV (51/60, 85%) and most HSIL was caused by hrHPV (80/86, 93%, $p<0.001$), making an important distinction between disease caused by lrHPV and by hrHPV.

Immunohistochemical staining patterns in lesions caused by lr- and hr-HPV infections

The relationship between expression patterns of p16 and E4, and causative HPV infection (lr- or hr-HPV) was explored in relation to the LAST classification. Of the lesions caused by lrHPV (57), most showed an extensive patchy p16 staining pattern (51/57, 90%) as shown in Figure 1, but 6/57, 11% showed diffuse p16 staining and were called HSIL. Lesions caused by hrHPV showed a diffuse p16 staining pattern in 96% (85/89) of cases (example in Figure 2), and the remaining 4 lesions showed a less extensive patchy staining pattern that was restricted to the lower one third of the epithelium. There was no significant difference in E4 positivity between lesions caused by lr- or hrHPV (E4 positivity: 22/57, 38.6% of lesions and 36/89, 40.4%, respectively, p=0.823).

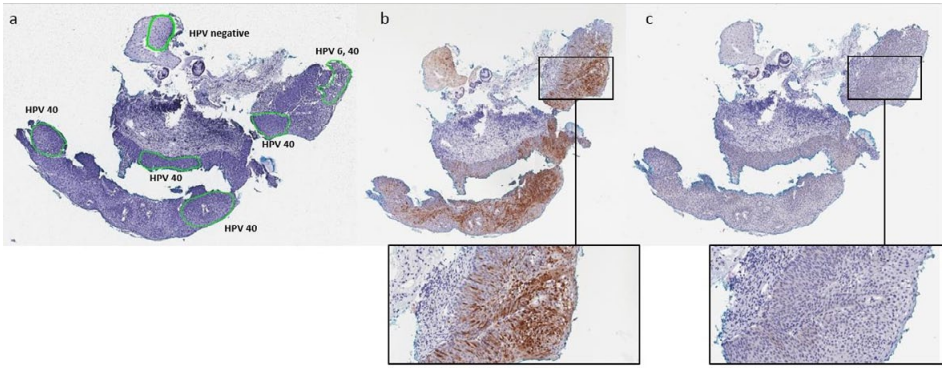

Figure 1: Consensus diagnosis AIN2/HSIL with genotypes HPV 6, 31 and 40 detected in the WTS. Six regions were selected for LCM-PCR and HPV40 was identified as the causative genotype of the worst lesion present (a). The p16 immunohistochemical stain shows an extensive patchy staining pattern (b, 20.0X magnification below) and there is no HPV E4 expression (c, 20.0X magnification below).

Figure 2: Consensus diagnosis AIN2/HSIL with genotypes HPV 16 and 18 detected in the WTS. Six regions were selected for LCM-PCR and HPV16 was identified as the causative genotype of the worst lesion present (a). The p16 immunohistochemical stain shows full thickness diffuse p16 staining (b, 20.0X magnification below) and there is extensive superficial HPV E4 expression (c, 20.0X magnification below).

Table 4 shows the relation between p16 grade and E4 positivity in HSIL. There was one HSIL/AIN3 that had a patchy p16 staining pattern, E4 negativity and was associated with lrHPV. In the group of 85 HSIL that showed diffuse p16 positivity (≥grade 2), there was a gradual decrease in E4 positivity from 60% (3/5) in HSIL showing p16 in the lower 1/3 of the epithelium, to 41% (9/22) of HSIL with p16 in the lower 2/3 and 35% (20/58) in HSIL with p16 in more than 2/3 of the epithelium.

Table 4: The relation between p16 grade and E4 positivity in HSIL.

p16 grade	E4		Total
	Negative	*Positive*	
1 - patchy	1 (100%)	0	1
2 - ≤lower 1/3	2 (40%)	3 (60%)	5
3 - ≤lower 2/3	13 (59%)	9 (41%)	22
4 - > lower 2/3	38 (66%)	20 (34%)	58
Total	54 (63%)	32 (37%)	86

DISCUSSION

This study showed that anal HSIL is heterogeneous, with p16 and E4 making complementary, specific contributions to defining the nature of an anal SIL. This enabled separation of HSIL into those expressing both HPV E4 and p16, and those only expressing p16. Increasing p16 expression was associated with decreasing E4 expression. This variation was partly reflected in the H/E grading of AIN2 and AIN3.

p16 is a surrogate marker for the activity of high-risk HPV E7 [26], which has transforming activity, but is also necessary for production of viral particles. Its grade increases with lesion severity both between LSIL and HSIL and with AIN grading. Its expression pattern can largely separate lrHPV infections from hrHPV infections. Importantly, there were two distinct extensive patterns of p16 staining, one being extensive patchy staining caused by lrHPV infection and the other being diffuse p16 staining caused by hrHPV infection. Recognizing the difference is important for accurate classification of lesions. Several studies have found similar differences in expression of p16 between lrHPV and hrHPV infection in cervical biopsies [27, 28]. Some patchy p16 expression is also seen in certain physiological states such as metaplasia in the cervix [29], but this is not as extensive as seen here.

E4 indicates the continued presence of completion of the life cycle of HPV [19], which is found in both LSIL and HSIL. We showed that hrHPV-positive HSIL with evidence of transformation as defined by diffuse p16 positivity are not homogeneous: almost half show evidence of continuing completion of the HPV life cycle and productive infection as indicated by E4 expression.

We showed that as p16 expression increases there was a decrease of E4 positivity with increasing p16 grade. Scoring of p16 and E4 IHC markers has previously been shown to be reproducible and separates productive from more advanced transforming infections

[18, 30]. This provides a more reliable potential approach to selecting patients for treatment than AIN/SIL diagnosis.

In this set of biopsies (176/183 from HIV+ MSM, 96.2%), few LSIL lesions (9/60, 15%) are associated with hrHPV, and only 6/86 (7%) of HSIL was associated with lrHPV. In the case of HIV+ MSM, the clinical implications of HSIL associated with lrHPV are uncertain [31]. Such HSIL/AIN2 lesions showing extensive patchy p16 staining in the absence of E4 might represent an abortive, non-productive low-risk HPV infection overexpressing HPV E7 and not completing the HPV life cycle. The mechanism for strong patchy expression of p16 in lrHPV infections is unclear [32].

This study supports the suggestion that anal HSIL represents a very heterogeneous group of lesions, consisting of productive lesions that are potentially self-limiting as well as transforming lesions with a risk of progression to cancer [14]. It also supports the use of p16 as a surrogate for hrHPV identification.

Scoring based on immunomarkers has a better inter- and intra-observer agreement compared to grading based on morphology [18, 33] and opens up the possibility of reproducible subclassification of the heterogeneity of HSIL. In our study, 37% of HSIL according to our LAST diagnosis showed E4 positivity as evidence of productive infections. Previous research in cervical intraepithelial neoplasia (CIN) showed that a dual biomarker approach using E4 and p16 can distinguish HPV-associated CIN1 from other pathologies and may be used to divide the CIN2 group according to the extent of life-cycle deregulation [17]. The percentage of E4 positive HSIL lesions (37%) is in line with the percentage of E4 positive CIN2 lesions that was found by van Baars et al. (43.5%) [18].

Based on the biomarker expressions used in this study, E4 positive hrHPV associated HSIL expressing p16 and lrHPV associated SIL with extensive patchy p16 warrant investigation of a "wait and see" management policy rather than immediate treatment. Studies of serial biopsies and well documented clinical follow-up studies are necessary to establish the optimal use of immunohistochemical markers in routine practice and to optimize patient selection for treatment.

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