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ARTICLE

Characterization of cervical biopsies of women with HIV and HPV co-infection using p16^{ink4a}, ki-67 and HPV E4 immunohistochemistry and DNA methylation

Wieke W. Kremer [ORCID][1] · Frederique J. Vink [ORCID][1] · Marjolein van Zummeren[1] · Greta Dreyer[2] · Lawrence Rozendaal[1] ·
John Doorbar[3] · Maaike C. G. Bleeker[1] · Chris J. L. M. Meijer[1]

Abstract

This study aims to characterize cervical intraepithelial neoplasia (CIN) in women living with HIV using biomarkers. Immunohistochemical (IHC) staining for human papillomavirus (HPV) E4 protein indicates CIN with productive HPV infection, whereas Ki-67 and p16^{ink4a} indicate CIN with transforming characteristics, which may be further characterized using DNA hypermethylation, indicative for *advanced* transforming CIN. Cervical biopsies ($n = 175$) from 102 HPV positive women living with HIV were independently reviewed by three expert pathologists. The consensus CIN grade was used as reference standard. IHC staining patterns were scored for Ki-67 (0–3), p16^{ink4a} (0–3), and E4 (0–2) and correlated to methylation levels of four cellular genes in corresponding cervical scrapes. Reference standards and immunoscores were obtained from 165 biopsies:15 no dysplasia, 91 CIN1, 31 CIN2, and 28 CIN3. Ki-67 and p16^{ink4a} scores increased with increasing CIN grade, while E4 positivity was highest in CIN1 and CIN2 lesions. E4 positive CIN1 lesions had higher Ki-67 and p16^{ink4a} scores and higher methylation levels compared with E4 negative CIN1 lesions. E4 positive biopsies with low cumulative Ki-67/p16^{ink4a} immunoscores (0-3) had significantly higher methylation levels compared with E4 negative biopsies. No significant differences in Ki-67 and p16^{ink4a} scores and methylation levels were observed between E4 negative and positive CIN2 or CIN3 lesions. The presence of high methylation levels in scrapes of CIN lesions with IHC characteristics of both productive (E4 positive) and transforming infections (increased Ki-67/p16^{ink4a} expression) in women living with HIV might indicate a rapid aggressive course of HPV infections towards cancer in these women.

Introduction

Cervical cancer is the fourth most common cancer diagnosed in women worldwide, mostly affecting low- and

✉ Chris J. L. M. Meijer
cjlm.meijer@amsterdamumc.nl

[1] Amsterdam UMC, Vrije Universiteit Amsterdam, Pathology, Cancer Center Amsterdam, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

[2] Department of Obstetrics and Gynecology, University of Pretoria, Pretoria, South Africa

[3] Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

middle-income countries, and one of the most common cancers in women living with human immunodeficiency virus (HIV) [1]. Cervical cancer is caused by a persistent infection with human papillomavirus (HPV) [2], and both are significantly more prevalent in women living with HIV compared with the general population [3–5]. Moreover, cervical cancer occurs at a younger age in women living with HIV, indicating earlier oncologic progression [6]. In South Africa, where HIV and HPV infection rates are among the highest worldwide, cervical cancer is the leading cause of cancer-related death in women [7].

Cervical cancer develops through preinvasive stages, referred to as cervical intraepithelial neoplasia (CIN, grade 1–3), which can be detected and treated via cervical screening. Histological classification of cervical lesions is based on morphological features in H&E stained slides alone and may be supported by p16^{inka4} and Ki-67 immunohistochemical (IHC) staining in difficult cases [8, 9]. Clinical management is based on the CIN grade. However,

grading of CIN lesions is only moderately reproducible due to the subjective interpretation of cellular abnormalities [10]. The majority of CIN1 lesions are associated with productive HPV infections, with a high regression rate (up to 60%) and a low progression risk to invasive cancer (about 1%), whereas most CIN3 lesions are associated with transforming infections, having a lower regression rate (about 30%) and a progression rate to invasive cancer of up to 30% [11, 12]. CIN2 lesions are particularly heterogeneous in both clinical behavior and chromosomal aberrations, including both transient productive and early transforming infections [13, 14]. Current clinicopathological classifications are unable to predict which lesions are likely to progress and, consequently, both CIN3 and CIN2 lesions are treated by ablative or excisional therapy in many countries. This results in considerable overtreatment and associated cervical morbidity [15]. The heterogeneity of CIN lesions can be shown by using IHC markers of productive HPV infection (HPV E4) and transforming HPV infection (Ki-67 and p16^{ink4a} overexpression as a result of HPV E7 activity) [16].

Another biomarker associated with transforming HPV infections and cervical cancer is methylation of promoter regions of host cell genes. Methylation-mediated silencing of host cell genes involved in cervical carcinogenesis, detected by methylation levels of promoter regions of such genes, has shown to increase with increasing CIN grade and methylation levels are very high in cervical cancer [14]. Moreover, several studies have shown that, in HIV-uninfected women, the presence of high methylation levels is associated with increasing chromosomal aberrations, similar to cervical cancer, suggesting that high methylation levels are associated with underlying lesions with a high short-term progression risk for cancer [17–19].

Few studies on Ki-67 and p16^{ink4a} expression and their diagnostic performance in women living with HIV have been published [20–22]. Moreover, studies evaluating the expression of HPV E4, indicating a productive HPV infection with a low short-term progression risk, and methylation of host cell genes, indicating a transforming infection with a high short-term progression risk for cervical cancer, in CIN lesions of women living with HIV are limited and integrated analysis of these markers has not been described for this population.

In this study we aim to characterize CIN lesions in women living with HIV using markers characteristic for transforming (i.e., Ki-67 and p16^{ink4a} expression) and productive infections (i.e., HPV E4 expression) In addition, we correlate the expression of these markers to the methylation levels of four host cell genes in the corresponding cervical scrapes in order to get insight in the aggressive nature of HPV-associated cervical lesions in women living with HIV.

Materials and methods

Study population

Clinical samples from a South African screening cohort of women living with HIV were used for this study. Ethical approval was granted by the Research Ethics Committee of the University of Pretoria (protocol numbers 100/2012 and 155/2014) and written informed consent was obtained from all participants. Detailed study procedures and baseline characteristics have been described previously [23–25].

From all study participants a liquid-based cytology sample was collected, followed by two colposcopy-directed biopsies from either the most abnormal area on the cervix or at random if no lesion was visible. Samples from women who tested HPV positive on liquid-based cytology using a clinically validated HPV PCR assay (GP5 + /6 + -PCR EIA and/or HPV-Risk assay) were included, in total 273 biopsies from 144 women. Figure 1 provides a schematic overview of the study procedures.

Immunohistochemistry

All formalin-fixed paraffin-embedded cervical biopsies were cut into seven sections of 3 um. The first and last sections were used for H&E staining to ensure presence of a lesion throughout all sections (sandwich technique). All biopsies were first classified by an expert pathologist (pathologist 1) based on morphologic characteristics as no dysplasia, CIN1, CIN2, CIN3, or invasive carcinoma (see paragraph 'H&E CIN grading'). All biopsies with an H&E CIN grade by pathologist 1 of CIN1 or worse (175 biopsies from 102 women in total) were stained for Ki-67, p16^{ink4a}, and HPV E4. In-between sections were used for immunostaining with mouse monoclonal antibodies against Ki-67 antigen (clone MIB-1, Dako, Agilent Technologies, USA) and p16^{ink4a} antigen (clone E6H4$^{™}$, CINtec$^{®}$, Roche, Switzerland) using the automated Ventana staining machine (Ventana Benchmark ULTRA, Ventana Medical Systems, Roche, USA). Furthermore, sections were also stained with the validated mouse monoclonal antibodies panHPVE4 (FH1.1., further referred to as E4, produced in the laboratory of dr. J. Doorbar [26] and kindly provided by him, reactive against HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, and 70), as described previously [27].

Scoring of cervical biopsies

All 175 biopsies of women who tested HPV positive on liquid-based cytology were reviewed by three expert pathologists (pathologist 1, 2, and 3) who were blinded to the HPV and methylation results from the cervical scrapes.

Fig. 1 Flowchart of study procedures. CIN cervical intraepithelial neoplasia; HPV+ high-risk human papillomavirus positive; H&E hematoxylin and eosin stain; IHC immunohistochemistry.
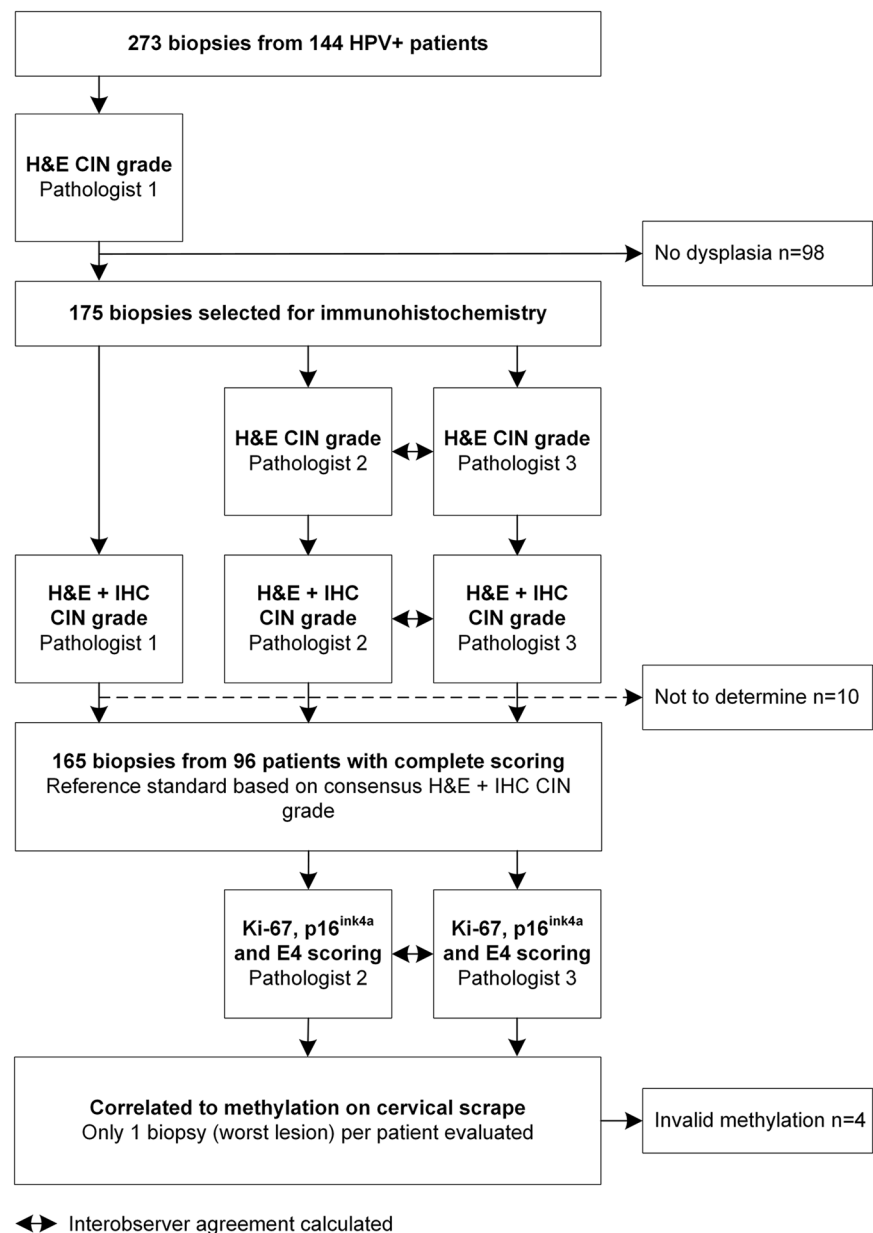
H&E CIN grading

The pathologists reviewed the H&E slides of the biopsies, selected the area with the most dysplastic epithelial features, and graded the lesion based on morphologic characteristics (further referred to as 'H&E CIN grade'). Biopsies were classified as no dysplasia, CIN1, CIN2, CIN3, or invasive carcinoma, according to international criteria [28].

Combined H&E and IHC CIN grading

The three pathologists then interpreted the Ki-67 and p16^{ink4a} expression in the area with the most dysplastic epithelial features, and generated a CIN grade based on morphologic

features combined with their interpretation of the Ki-67 and/or p16^{ink4a} immunostainings (further referred to as 'combined H&E and IHC CIN grade'). The consensus diagnosis of the combined H&E and IHC CIN grade based on agreement in at least two out of three pathologists, was used as the 'reference standard'. If no majority agreement was reached, consensus was achieved in a panel discussion (in total 14 biopsies).

Scoring of Ki-67, p16^{ink4a}, and E4

Two pathologists (pathologist 2 and 3) scored the expression of Ki-67 (score 0–3), p16^{ink4a} (score 0–3), and E4 (score 0–2) in the most dysplastic area, as described previously [16, 27]. In brief, no increased Ki-67 nuclear staining, i.e., only

Table 1 Expression of Ki-67 (A), p16ink4a (B), and E4 (C) across CIN grades.

Reference standard								
(A)	Ki-67 scores							
	0		1		2		3	
	n	%	n	%	n	%	n	%
No dysplasia (n = 15)	7	46.7%	8	53.3%	0	0%	0	0%
CIN1 (n = 91)	10	11.0%	73	80.2%	8	8.8%	0	0%
CIN2 (n = 31)	0	0%	2	6.5%	22	71.0%	7	22.6%
CIN3 (n = 28)	0	0%	1	3.6%	8	28.6%	19	67.9%
(B)	p16ink4a scores							
	0		1		2		3	
	n	%	n	%	n	%	n	%
No dysplasia (n = 15)	15	100%	0	0%	0	0%	0	0%
CIN1 (n = 91)	72	79.1%	8	8.8%	11	12.1%	0	0%
CIN2 (n = 31)	1	3.2%	5	16.1%	21	67.7%	4	12.9%
CIN3 (n = 28)	0	0%	0	0%	8	28.6%	20	71.4%
(C)	E4 scores							
	0		1		2			
	n	%	n	%	n	%		
No dysplasia (n = 15)	13	86.7%	1	6.7%	1	6.7%		
CIN1 (n = 91)	57	62.6%	15	16.5%	19	20.9%		
CIN2 (n = 31)	9	29.0%	10	32.3%	12	38.7%		
CIN3 (n = 28)	19	67.9%	7	25.0%	2	7.1%		

Ki-67 scores: (0) normal nuclear staining of basal epithelial layer; (1) staining of the lower one-third of the epithelium; (2) staining of lower two-thirds of the epithelium; (3) staining of more than two-thirds of the epithelium. P16ink4a scores: (0) negative or patchy staining; (1) diffuse staining of the lower one-third of the epithelium; (2) staining of lower two-thirds of the epithelium; (3) staining of more than two-thirds of the epithelium. E4 scores: (0) negative staining; (1) staining restricted to the upper quarter of the epithelium (focal); (2) staining in the upper one-third of the epithelium or more (extensive).

CIN cervical intraepithelial neoplasia.

staining of cells in the basal layer was scored 0, and increased Ki-67 staining up to the lower one-third, two-thirds, or more than two-thirds of the epithelium was scored as 1, 2, or 3, respectively. Negative or *patchy* (i.e., non-diffuse) p16ink4a staining was scored as 0 and diffuse staining up to the lower one-third, two-thirds, or more than two-thirds was scored as 1, 2, or 3, respectively. Using these scores, a cumulative Ki-67/p16ink4a immunoscore (0–6) was generated for each biopsy. E4 expression was scored as negative (0) in the absence of E4 positive epithelial cells, E4 positivity restricted to upper quarter of the epithelium was scored as focal [1], and E4 positivity in the upper one-third of the epithelium or more was scored as extensive [2].

Methylation analysis

Methylation analysis by quantitative methylation specific PCR (qMSP) on bisulfite converted DNA from cervical scrapes collected at baseline was performed previously [24, 29]. In this study, host cell genes *ASCL1*, *LHX8*, *FAM19A4*, and *miR124-2*, involved in cervical carcinogenesis, were evaluated. Methylation values off all targets were normalized to the reference gene B-actin (ACTB) and the calibrator using the comparative Ct method ($2^{-\Delta\Delta Ct} \times 100$), resulting in ΔΔCt ratios [30].

Statistical analysis

Kappa statistics were used to assess the interobserver agreement between the different scoring methods (i.e., H&E CIN grade, combined H&E and IHC CIN grade, Ki-67 score, p16ink4a score, Ki-67/p16ink4a immunoscore, and E4 score) of the cervical biopsies of two pathologists (2 and 3), as they completed all scoring methods. Quadratic weighted kappa was calculated using the different categories of each scoring method and interpreted according to the standards of Landis and Koch [31].

Fig. 2 E4 staining patterns in CIN lesions. a CIN1 lesion (ID45) with extensive E4 staining (score 2). Corresponding Ki-67 and p16 stainings showed Ki-67 positivity staining up to the lower one-third of the epithelium (score 1) and patchy p16 staining (score 0). Hypermethylation was found for three methylation markers. **b** CIN2 lesion (ID67) with extensive E4 staining (score 2). Corresponding Ki-67 and p16 stainings showed increased Ki-67 staining up to the lower two-third of the epithelium (score 2) and diffuse p16 staining up to the lower one-third (score 1) of the epithelium. Hypermethylation was found for three methylation markers. **c** CIN 3 lesion (ID90) with focal E4 staining (score 1). Corresponding Ki-67 and p16 stainings showed full thickness Ki-67 staining of the epithelium (score 3) and full thickness p16 staining of the epithelium (score 3). Hypermethylation was found for 3 methylation markers. ID numbers correspond with the ID numbers of Fig. 3.
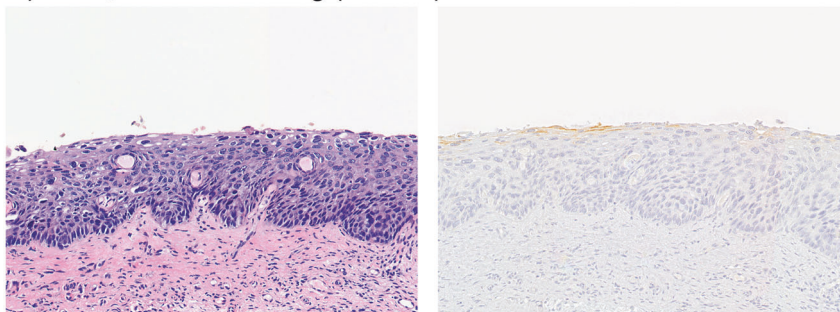

A) CIN1, extensive E4 staining (score 2)
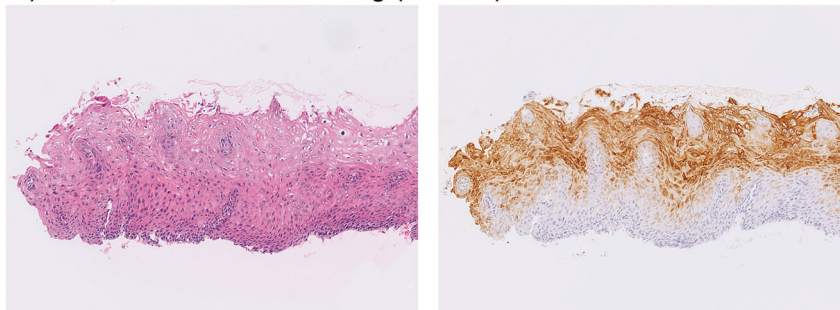
B) CIN2, extensive E4 staining (score 2)
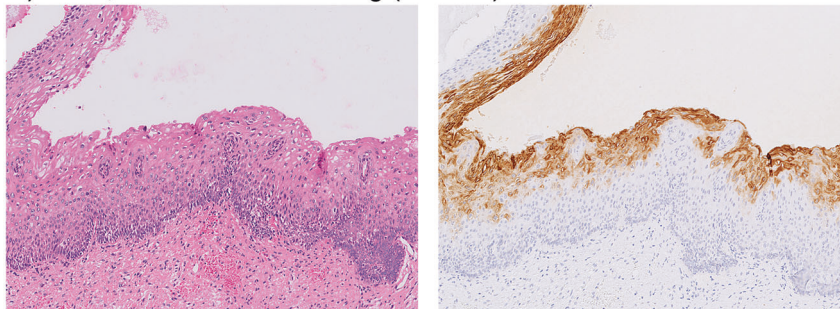
C) CIN3, focal E4 staining (score 1)

Absolute and proportional scoring results of Ki-67, $p16^{ink4a}$, and E4 from each pathologist were calculated for all biopsies and stratified by CIN grade based on the reference standard. Ki-67 and $p16^{ink4a}$ expression was compared between E4 negative and E4 positive (i.e., focal or extensive) biopsies, stratified by the reference standard, using Fisher's exact analysis.

Methylation levels in cervical scrapes were correlated to the highest reference standard present in the corresponding biopsy of the same patient and, in case of two biopsies with the same reference standard, to the highest Ki-67/$p16^{ink4a}$ immunoscore, as the methylation levels are considered to represent the worst underlying cervical lesion [32]. The Mann–Whitney U testing was used to compare methylation levels in lesions with different Ki-67/$p16^{ink4a}$ and E4 expression patterns.

All statistical analyses were performed using SPSS (V.22) and STATA (V.14), and p values of 0.05 or less were considered statistically significant.

Results

In total 165 biopsies of 96 HPV positive patients had adequate H&E and IHC staining and were included in the analyses (Fig. 1). From these biopsies a reference standard was obtained, resulting in 15 biopsies with no dysplasia, 91 CIN1, 31 CIN2, and 28 CIN3.

Interobserver agreement

The results of each scoring method are presented in Supplementary Table 1. According to the standards of Landis and Koch [31], the different scoring methods had substantial to almost perfect agreement between pathologists 2 and 3, with weighted kappa values of 0.70, 0.74, 0.82, 0.84, and 0.82 for H&E CIN grade, combined H&E and IHC CIN grade, Ki-67, $p16^{ink4a}$, and E4 scoring, respectively. Furthermore, the cumulative Ki-67/$p16^{ink4a}$ immunoscore had the highest agreement (weighted kappa 0.87). Because of

this high interobserver agreement, only the scoring results of one pathologist (pathologist 3) are reported. Similar results were obtained when the scoring results of pathologist 2 were evaluated.

Expression patterns of Ki-67, p16[ink4a], and E4 in cervical biopsies

Table 1 provides an overview of the expression of Ki-67, p16[ink4a], and E4 stratified by reference standard. In biopsies with no dysplasia, Ki-67 and p16[ink4a] expression was normal in 46.7% and 100%, respectively, while in biopsies with CIN3, Ki-67, and p16[ink4a] were expressed in more than two-thirds of the lower epithelial layer in 67.9% and 71.4%, respectively. E4 positivity was mostly observed in CIN1 and CIN2 lesions: 37.4% of CIN1 and 71.0% of CIN2 were positive for E4 (16.5% and 32.3% with focal E4 expression (score 1), 20.9% and 38.7% with extensive E4 expression (score 2) in CIN1 and CIN2, respectively). Positive E4 staining was observed in 32.1% of CIN3 lesions, but this was mostly focal expression (25% focal, 7.1% extensive). Figure 2 shows examples of a CIN1 and CIN2 lesion with extensive E4 staining pattern and a CIN3 lesion with focal E4 expression.

Ki-67 and p16[ink4a] expression was compared between biopsies with productive (focal (score 1) or extensive E4 staining (score 2), E4 positive), and nonproductive (no E4 staining (score 0), E4 negative), characteristics (Table 2). E4 positive CIN1 lesions had higher Ki-67 and p16[ink4a] scores compared with E4 negative CIN1 lesions (p values < 0.001 and 0.006 for Ki-67 and p16[ink4a], respectively). No significant differences in Ki-67 and p16[ink4a] scores were observed between E4 negative and E4 positive CIN2 or CIN3 lesions (p values > 0.1).

Correlation immunohistochemical findings in cervical biopsies with methylation levels in cervical scrapes

The expression patterns of E4, Ki-67, and p16[ink4a] in cervical biopsies and methylation of *FAM19A4*, *miR124-2*, *ASCL1*, and *LHX8* in the corresponding cervical scrapes are shown in Fig. 3. This figure illustrates not only the increase in Ki-67 and p16[ink4a] expression and methylation levels with increasing CIN grade, but also the heterogeneity in CIN lesions. Figure 4 shows the distribution of methylation levels of all genes evaluated in E4 negative and E4 positive biopsies, stratified into low (0–3) and high (4+) cumulative Ki-67/p16[ink4a] immunoscores. E4 positive biopsies with low Ki-67/p16[ink4a] immunoscores had significantly higher methylation levels compared with E4 negative biopsies with low Ki-67/p16[ink4a] immunoscores. Methylation levels in cervical scrapes corresponding to biopsies with high Ki-67/p16[ink4a] immunoscores did not differ between E4 positive and E4 negative biopsies. When stratified by reference standard (i.e., CIN1 or less, CIN2, and CIN3), similar

Table 2 Ki-67 and p16[ink4a] expression in cervical biopsies with productive (E4 positive) and nonproductive (E4 negative) characteristics.

| | Ki-67 scores | | | | | | | | p16[ink4a] scores | | | | | | | |
| | 0 | | 1 | | 2 | | 3 | | 0 | | 1 | | 2 | | 3 | |
Reference standard	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
No dysplasia																
E4 negative ($n = 13$)	6	46.2%	7	53.8%	0	0%	0	0%	13	100%	0	0%	0	0%	0	0%
E4 positive ($n = 2$)	1	50.0%	1	50.0%	0	0%	0	0%	2	100%	0	0%	0	0%	0	0%
CIN1																
E4 negative ($n = 57$)	10	17.5%	47	87.0%	0	0%	0	0%	50	87.7%	1	1.8%	6	10.5%	0	0%
E4 positive ($n = 34$)	0	0%	26	76.5%	8	23.5%	0	0%	22	64.7%	7	20.6%	5	14.7%	0	0%
CIN2																
E4 negative ($n = 9$)	0	0%	1	11.1%	6	66.7%	2	22.2%	1	11.1%	0	0%	6	66.7%	2	22.2%
E4 positive ($n = 22$)	0	0%	1	4.5%	16	72.7%	5	22.7%	0	0%	5	22.7%	15	68.2%	2	9.1%
CIN3																
E4 negative ($n = 19$)	0	0%	1	5.3%	5	26.3%	13	68.4%	0	0%	0	0%	4	21.1%	15	78.9%
E4 positive ($n = 9$)	0	0%	0	0%	3	33.3%	6	66.7%	0	0%	0	0%	4	44.4%	5	55.6%

Ki-67 scores: (0) normal nuclear staining of basal epithelial layer; (1) staining of the lower one-third of the epithelium; (2) staining of lower two-thirds of the epithelium; (3) staining of more than two-thirds of the epithelium. P16[ink4a] scores: (0) negative or patchy staining; (1) diffuse staining of the lower one-third of the epithelium; (2) staining of lower two-thirds of the epithelium; (3) staining of more than two-thirds of the epithelium. E4 positive staining includes both focal (score 1) and extensive (score 2) staining.

CIN cervical intraepithelial neoplasia.

LEGEND

	negative (0)	focal (1)	extensive (2)	
E4	negative (0)	focal (1)	extensive (2)	
Ki-67	basal (0)	<lower 1/3 (1)	lower 1/3 to 2/3 (2)	>lower 2/3 (3)
p16ink4a	negative (0)	<lower 1/3 (1)	lower 1/3 to 2/3 (2)	>lower 2/3 (3)
FAM19A4	≤ 25th percentile	>25th ≤ 50th percentile	>50th ≤ 75th percentile	>75th percentile
miR124-2	≤ 25th percentile	>25th ≤ 50th percentile	>50th ≤ 75th percentile	>75th percentile
ASCL1	≤ 25th percentile	>25th ≤ 50th percentile	>50th ≤ 75th percentile	>75th percentile
LHX8	≤ 25th percentile	>25th ≤ 50th percentile	>50th ≤ 75th percentile	>75th percentile
MM+*	0 markers positive	1 marker positive	2 markers positive	3 markers positive

Fig. 3 Expression patterns of E4, Ki-67, and p16ink4a immunohistochemistry in cervical biopsies, and methylation of *FAM19A4*, *miR124-2*, *ASCL1*, and *LHX8* in HPV positive cervical scrapes. This figure shows the immunohistochemical scores for E4 (0–2), Ki-67 (0–3), and p16ink4a (0–3) in cervical biopsies across histological subgroups based on the reference standard, and methylation levels (ΔΔCt ratios) in corresponding cervical scrapes. In each subgroup, samples are consecutively ordered low-high based on their E4, Ki-67, p16ink4a, and methylation scores. The colors refer to the extent of immunohistochemical staining and hypermethylation, as indicated in the Legend. Methylation levels were categorized into ≤25th percentile, >25th ≤ 50 percentile, >50th ≤ 75 percentile, and >75th percentile; percentiles were calculated from all evaluated cervical scrapes. Each column within a subgroup represents one case: 6 no dysplasia, 45 CIN1, 23 CIN2, 18 CIN3. HPV human papillomavirus, CIN cervical intraepithelial neoplasia, MM methylation markers. *Positivity for either *FAM19A4* and/or *miR124-2* was caunted as one marker as this is a clinically validated kit.

results were obtained: E4 positive biopsies with CIN1 or less had significantly higher methylation levels compared with E4 negative biopsies with CIN1, but no differences in methylation levels were observed between E4 positive and E4 negative biopsies with CIN2 or CIN3 (see Supplementary Fig. 1).

Discussion

In this study we described IHC staining patterns of Ki-67, p16ink4a, and HPV E4 in a series of cervical biopsies from HIV and HPV co-infected women from a South African screening cohort. The extent of epithelial expression of Ki-67 and p16ink4a, markers of a transforming HPV infection, increased with severity of cervical dysplasia, while E4 expression, a marker for productive infection, increased from CIN1 (37%) to CIN2 (71%) and decreased in CIN3 (32%, mostly focal). This abundant expression of E4 in this population indicates more production of infectious virions and a high proportion of productive HPV infections compared with CIN lesions from HIV-uninfected women [26, 27]. Productive CIN1 lesions (i.e., E4 positive) showed moderately increased expression of Ki-67 and p16ink4a, and increased methylation of host cell genes in the corresponding cervical scrape compared with nonproductive

Fig. 4 Methylation levels in productive and non-productive cervical lesions. Methylation levels of *FAM19A4* (**a**), *miR124-2* (**b**), *ASCL1* (**c**), and *LHX8* (**d**) in cervical scrapes corresponding to biopsies with productive (E4 positive) and nonproductive (E4 negative) characteristics, stratified by Ki-67/p16^{ink4a} immunoscore.
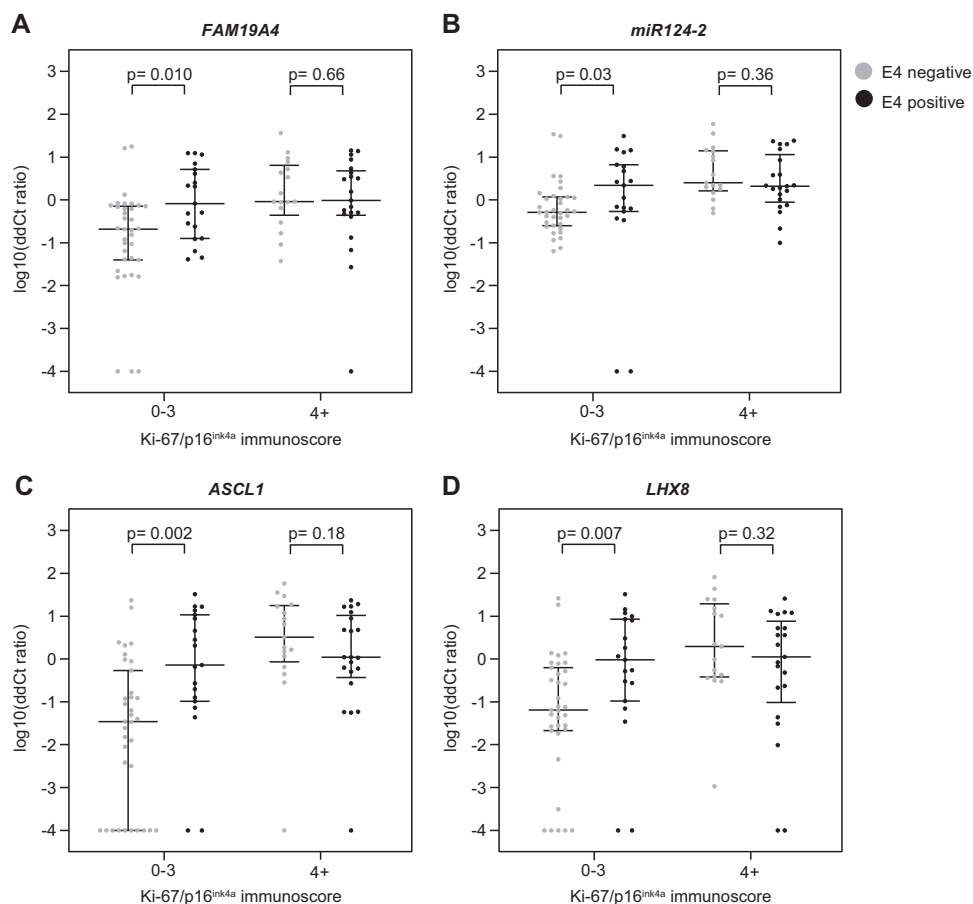
(i.e., E4 negative) CIN1 lesions. Together these findings illustrate the heterogeneity of CIN lesions in women living with HIV and show that these cervical lesions can exhibit productive and transforming characteristics simultaneously.

This simultaneous expression of markers indicative for productive and transforming infections may be the result of the immunocompromised status of women living with HIV, resulting in decreased HPV clearance, increased susceptibility for HPV-induced cellular transformation, and faster progression to cancer. This lack of immune control may also lead to increased expression of HPV E6 and E7 oncogenes, reflected by the presence of Ki-67, p16^{ink4a}, and hypermethylation of host cell genes, and cause cellular transformation. Viral oncogene expression may be further aggravated by a molecular interaction between HIV and HPV. It has been suggested that the HIV protein *tat* contributes to HPV-induced carcinogenesis by stimulating the expression of E6 and E7 [33, 34] and by increasing methylation of host cell genes through upregulation of DNA methyltransferase expression [35]. Alternatively, the E4 negative CIN1 lesions may not be representative of the high-risk HPV infection detected in the corresponding cervical scrape due to a sampling error in taking the cervical biopsies. Unfortunately, due to insufficient quality of the

DNA isolated from the cervical biopsies, we were unable to detect HPV directly in the CIN lesion. Other explanations for the lack of E4 expression in CIN1 lesions include tangential sectioning and tissue damage during sample processing, causing loss of the upper epithelial layer expressing E4. Furthermore, some E4 negative CIN1 lesions may represent regressive lesions [36]. Future studies to investigate the expression of HPV E4 in CIN1 lesions are indicated. A schematic representation of the expression of the IHC and methylation markers in women living with HIV and previous findings in HIV-uninfected women [27] is given in Fig. 5.

To our knowledge, this is the first study describing the expression of E4 in cervical biopsies from women living with HIV. In a similar series from HIV-uninfected women, Leeman et al. describe expression of p16^{ink4a} and E4 in cervical biopsies and methylation of *FAM19A4* and *miR124-2* genes in corresponding cervical scrapes [37]. Although E4 was less expressed in their series, E4 positivity was highest in CIN1 and CIN2 lesions and was associated with the presence of p16^{ink4a} positivity up to two-thirds of the epithelium. This is consistent with our observation that E4 positive CIN1 lesions have higher Ki-67 and p16^{ink4a} scores compared with E4 negative CIN1 lesions and may be
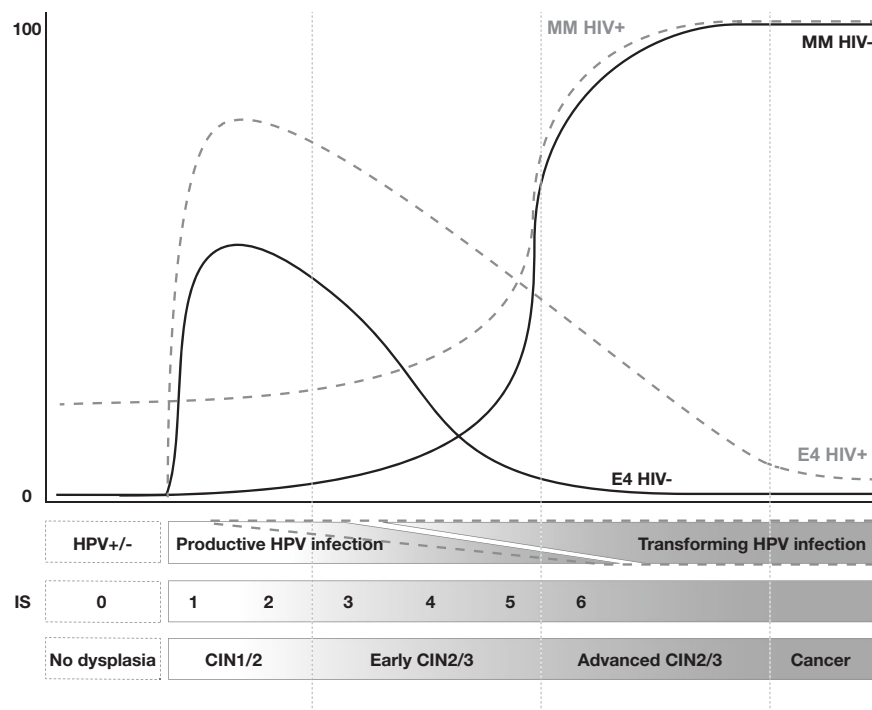
Fig. 5 Concept of cervical carcinogenesis and changes in E4 expression and methylation of host cell genes in HIV-infected (grey dotted lines) and HIV-uninfected (black lines) women. Productive (E4 expression) and transforming (Ki-67/p16^{ink4a} expression and hypermethylation) characteristics show greater overlap in HIV-infected women compared with HIV-uninfected women [27], suggesting that the process of transformation starts earlier after an HPV infection in HIV-infected women compared with HIV-uninfected women, which could be consistent with a faster progression of CIN lesions to cancer in HIV-infected women. Methylation levels of host cell genes are higher in HIV-infected women without dysplasia or cervical intraepithelial neoplasia (CIN) grade 1 compared with HIV-uninfected women. More E4 positivity is present in HIV-infected compared with HIV-uninfected women. CIN cervical intraepithelial neoplasia, HIV human immunodeficiciency virus, HPV human papillomavirus, IS immunoscore, MM methylation markers.

explained by the E7-driven epithelial proliferation above the basal layer, which is necessary for viral reproduction [38].

In line with previous reports on the value of p16^{ink4a} and Ki-67 immunohistochemistry in the diagnosis of CIN [8, 9, 16, 20, 39], our study suggests that these markers could be used to optimize identification of CIN lesions in women living with HIV requiring treatment. However, the question remains whether there is a role for any of these IHC markers beyond the objective identification of CIN. Indeed, the prognostic value of Ki-67 and p16^{ink4a} in the clinical behavior of CIN2 and CIN3 lesions has shown to be limited [40–42] and the clinical value of E4 remains to be determined. Host cell methylation on the other hand, is a promising biomarker as increased methylation of host cell genes is associated with more chromosomal aberrations necessary for progression to cervical cancer [17–19]. In fact, the prognostic value of methylation analysis was recently demonstrated by Louvanto et al. showing that methylation of cellular gene *EPB41L3* is associated with progression of untreated CIN2 in HIV-uninfected women [43]. The same marker was also evaluated in African women living with HIV showing increased methylation in women with progressive lesions [44].

Together these data support the notion that the presence of hypermethylation of host cell genes indicates an advanced transforming lesion with a high short-term progression risk, and therefore could be used to direct treatment decisions. In women living with HIV, high baseline methylation levels [24, 25] might illustrate their high cervical cancer progression risk compared with HIV-uninfected women. Clinical follow-up studies are now in progress to evaluate the prognostic value of methylation analysis in women living with HIV, and to show whether all women living with HIV with high methylation levels indeed require treatment of these lesions. In addition, further evaluation of genetic and epigenetic alterations associated with cervical cancer in women living with HIV is required to support our hypothesis that these lesions have an increased progression risk.

Strong points of our study are that we have thoroughly reviewed all cervical dysplastic features within HIV and HPV co-infected women and that a histological endpoint was available for all women screened, thereby creating the possibility to analyze the full spectrum of HPV-associated cervical disease. A limitation of this study is that the duration of HPV infection associated with the lesion cannot be estimated,

as most women participating had never been screened before. Therefore, long-existing lesions may be present in this under-screened population and may provide an explanation for the high methylation levels found in our study.

Another limitation is that some biopsies may not represent the worst underlying lesion due to a sampling error in taking the biopsy. It should, however, be realized that excisional treatment of the transformation zone of women in this study was not only based on a CIN2+ finding in the cervical biopsy, but also directly on a high-grade squamous intraepithelial lesion or worse (≥HSIL) cytology result [25]. This study algorithm results in a small group of women having ≤CIN1 on cervical biopsy with a CIN3 in their excision specimen, suggesting a possible sampling error (see Supplementary Table 2). Unfortunately, the excision specimens were not available for IHC evaluation. We accounted for this sampling error by repeating the analyses after exclusion of women with a CIN3+ diagnosis on their excision specimen that was not diagnosed in the biopsy. The same trend of higher methylation levels in cervical scrapes of women with E4 positive low-grade lesions compared with E4 negative low-grade lesions was observed, yet only significant for *ASCL1*.

Conclusion

In this series of cervical biopsies from HIV and HPV co-infected women, CIN lesions with both productive (E4 positivity) and transforming characteristics (increased Ki-67 and p16^{ink4a} staining) were abundantly present. The presence of high methylation levels in scrapes of CIN lesions with IHC characteristics of productive and/or transforming infections in women living with HIV could suggest a rapid progression of CIN lesions towards cancer in these women.

Compliance with ethical standards

Conflict of interest CJLMM is a minority shareholder of Self-screen B.V., a spin-off company of VUmc which develops, manufactures and licenses the high-risk HPV assay and methylation marker assays for cervical cancer screening and holds patents on these tests. CJLMM is part-time CEO of Self-Screen B.V., has a very small number of shares of Qiagen and MDXHealth, has received speakers' fees from GSK, Qiagen, and SPMSD/Merck, and served occasionally on the scientific advisory boards (expert meeting) of these companies. WWK, FJV, MZ, MCGB, LR, JD and GD declare no conflicts of interest.

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