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Endocervical adenocarcinomas and HPV genotyping in an HIV endemic milieu – a retrospective study

Lucília Lovane[1,2,3*], Gabriella Lillsunde Larsson[4], Satish Tulsidás[5], Carla Carrilho[1,3], Sören Andersson[2] and Christina Karlsson[4]

Abstract

Background Cervical cancer is the most prevalent cancer in Mozambique, with endocervical adenocarcinoma accounting for approximately 5.5% of cases. Knowledge regarding the most prevalent HPV genotypes in endocervical adenocarcinoma is limited, within this setting. This study aimed to investigate human papillomavirus (HPV) prevalence and genotypes within a cohort of endocervical adenocarcinoma patients in the context of Mozambique's recently introduced vaccination programme, considering the country's HIV-endemic setting.

Methods Forty consecutive cases of endocervical adenocarcinoma diagnosed at Maputo Central Hospital between 2017 and 2018, with limited clinical data available, were included. Human immunodeficiency virus (HIV) status was determined through serological data or in situ hybridisation on histopathological slides. HPV detection was performed using a multi-methodological approach, including Anyplex II, in-house polymerase chain reaction (PCR), and chromogenic and fluorescent in situ hybridisation techniques.

Results All 40 cases exhibited HPV-dependent morphology. Fourteen of the 40 patients were HIV-positive. No significant differences were observed between the two groups regarding age, stage, or histopathological type. hrHPV16, 18, or 45 were detected in all cases. Notably, multiple hrHPV infections were identified exclusively in HIV-negative cases (10/26, $p=0.0075$), with hrHPV18/45 co-infection being the most common ($n=8$).

Conclusions These findings suggest that the newly implemented quadrivalent vaccination programme has the potential to prevent morbidity and mortality from endocervical adenocarcinoma, irrespective of HIV infection status, in Mozambique's HIV-endemic environment.

Keywords Endocervical adenocarcinomas, HPV, HIV, In situ hybridisation, HPV vaccine

*Correspondence:
Lucília Lovane
lucilia.trindade@gmail.com; lucilia.matias@oru.se
[1] Pathology Department, Maputo Central Hospital, Avenida Agostinho Neto 164, Maputo CEP1100, Mozambique
[2] School of Medical Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden
[3] Faculty of Medicine, Eduardo Mondlane University, Maputo, Mozambique
[4] School of Health Sciences, Faculty of Medicine and Health, Örebro University, Örebro, Sweden
[5] Medical Oncology Service, Maputo Central Hospital, Maputo, Mozambique

Background

Despite the reported decline in cervical cancer (CC) incidence in developed countries with well-established prevention screening programmes introduced in the late 1960s, as well as more recent HPV-based screening and HPV vaccination [1–4], CC remains the fourth most frequent cancer and a significant cause of cancer-related death in women globally, with an estimated 662 300 new cases and 348 900 deaths in 2022 [5–7]. The majority of these cases occur in low- and middle-income countries (LMICs) such as sub-Saharan Africa, which has the highest incidence and mortality rates [7]. Squamous cell carcinoma (SCC) is the most prevalent histological type of CC, and endocervical adenocarcinomas (EACs) the second most common type representing approximately 20–25% of all CC cases [8, 9]. While most CCs are caused by high-risk human papillomavirus (hrHPV), approximately 5% of SCCs are HPV-negative [10]. However, EACs present a different profile, with around 15% of cases being HPV-negative [11–13]. These HPV-negative EACs exhibit distinct aetiologies, molecular features, and morphological characteristics [8, 9, 14, 15]. The World Health Organization (WHO) 2020 classification of female genital tumours classifies EACs into two categories based on morphological patterns and aetiology. Morphological patterns are defined by cytoplasmic characteristics, the presence of easily identified apical mitoses, and/or apoptotic bodies as either HPV-associated or HPV-independent [8, 11, 15, 16].

In Mozambique, a southern African country, CC is the leading cause of cancer and cancer-related mortality [17]. The country has a high prevalence of HPV in the general female population (23.7–66.7%), which is even higher among women living with HIV (WLWH) (39.2–79%) [18–20]. The national HIV prevalence is 13% [21, 22].

HIV infection is known to contribute to persistent HPV infection and infection with multiple HPV types [4]. Additionally, WLWH are at increased risk of developing CC compared to HIV-negative women due to immunosuppression [4, 23, 24].

Similar to other sub-Saharan and LMICs, Mozambique's national screening programme has adopted the Visual Inspection with Acetic Acid (VIA) followed by cryotherapy or thermal ablation [25, 26]. Cytology screening through Pap smears is opportunistic and has low coverage [27]. The quadrivalent HPV vaccine (HPV6/11/16/18) was introduced into the national vaccination schedule for 9-year-old girls in November 2021 as a primary prevention measure [28]. A recent study reported an EAC prevalence of approximately 5.5% in Mozambique [29].

However, knowledge about HPV prevalence and genotypes in EAC among the HIV-endemic population, and the potential impact of the quadrivalent HPV vaccine, remains limited.

This study aims to characterise the histopathological features, including p16 immunohistochemistry, and determine the frequency and genotypes of HPV in EAC cases diagnosed at a referral university hospital in Maputo, Mozambique.

Methods

Sample collection and processing

This is a retrospective observational study performed in CC tissue sample material stored in Formalin-fixed paraffin-embedded (FFPE) blocks in the department of Pathology of Maputo Central Hospital (MCH), Mozambique from January 2017 to December 2018. The cases were identified and retrieved from the electronic files from computer databases of the department of pathology and hospital cancer registry at MCH**,** of the over 700 CC cases diagnosed during this period, 46 were diagnosed as EAC. Included in this study were, 40 cases of EAC consecutively diagnosed in pathology department of MCH, with sufficient tissue material for tissue microarrays (TMAs) construction and extraction for molecular analysis. The FFPE were retrieved from the biobank of the pathology department of MCH, and the blocks were transferred to Örebro university through a material transfer agreement. The remaining 6 cases with insufficient material were excluded. Demographic, clinical and HIV serology data were collected from the hospital's electronic pathology department database and hospital cancer registry databases of the Maputo Central Hospital.

New sections from all FFPE tissue blocks were cut and stained with haematoxylin and eosin (H&E). Slides were scanned (Pannoramic 250, 3D Histech Ltd, Hungary), assessed, and three 0.6 mm diameter areas of interest were selected for TMA construction using an automated TMA Grand master (3D Histech Ltd, Hungary) at the Department of Clinical Research of Orebro University Hospital (OUH), Sweden, and stored within a storage system from the same supplier at a server facility, fulfilling the general data protection regulation.

Immunohistochemistry

Immunohistochemistry for p16 (clone E6H4), a surrogate HPV biomarker, was performed using an automated platform (Ventana Roche, Roche diagnostics, Switzerland) according to the manufacturer's protocol. Slides were scanned as described above. Positive staining was defined as cytoplasmic and nuclear brown staining. p16 expression was scored on a four-grade scale: 3 + (strong diffuse), 2 + (moderate intensity), 1 + (weak or sporadic), and 0 (no staining).

Molecular analysis of HPV and HIV

DNA was extracted from FFPE tissue from one cylinder of 1 mm diameter that was extracted from the block using a sterile needle, using the manufacturer's protocol (Qiagen, Germany). HPV detection was performed using the Anyplex II HPV 28 assay (Seegene, Korea), as previously described [30, 31]. Cases with negative or invalid results were retested using an in-house real-time PCR targeting E6 or E7, as well as 12 high-risk and 2 low-risk HPV genotypes [30].

For cases remaining negative or invalid after PCR, RNA in situ hybridisation (ISH) was performed using RNAscope® 2.5 HD Detection Reagent Brown and multiplex fluorescent reagent Kit v2 (Advanced Cell Diagnostics, Inc., Newark, CA), as previously described [32]. Chromogenic ISH (CISH) was conducted on whole tissue sections using a cocktail of 18 HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). Positive and negative control probes were included (Hs-PPIB REF#313901 and DapB REF#310043 respectively). CISH slides were scanned as previously described, with positive cases showing brown punctate dots in the cytoplasm and/or nucleus [33, 34].

Subsequently, fluorescent ISH (FISH) targeting hrHPV16, 18, and 45 was performed on whole tissue sections. Positive and negative control tissues were included. Multiplex fluorescence detection was labelled with Opal 520, Opal 570, and Opal 690, with nuclei counterstained with DAPI. FISH slides were scanned on Pannoramic Midi (3D Histech Ltd, Hungary). The stepwise analysis of HPV testing is outlined in Fig. 1.

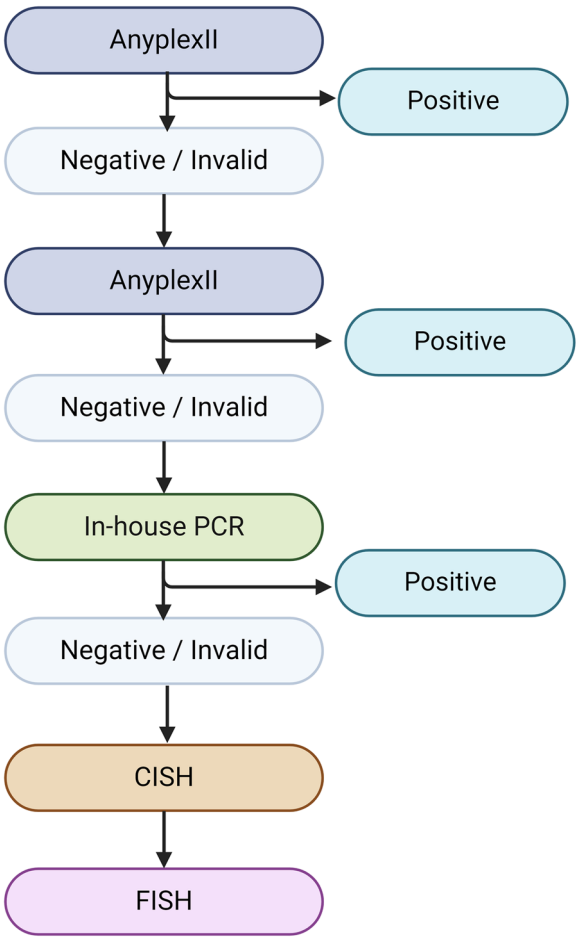

Fig. 1 Illustration of the stepwise HPV testing strategy for endocervical adenocarcinomas

HIV status determination

HIV status was known for 18 of the 40 cases based on serological data. For the remaining 22 cases, HIV status was determined using RNAscope® 2.5 assay (Advanced Cell Diagnostics, Inc., Newark, CA), as described above. The samples were considered to have specific staining signals when a brown single or two dots were identified in the nucleus [35, 36]. Positive and negative controls were included.

Statistical analysis

Age comparison between HIV-negative women and WLWH was performed using the Mann–Whitney U-test. Fisher's exact test was used for group comparisons of morphological types and multiple infections, while the Chi-two test was applied for p16 and HPV genotyping data. Two-tailed p-values < 0.05 were considered significant. Statistical analyses were performed using SPSS (IBM SPSS 28.0.0.0).

Results

Data related to age, morphology, and p16 expression are summarised in Table 1.

A total of 40 cases of EAC were included in the study. HIV status was determined for all 40 cases, with 14/40 (35%) being WLWH (7 cases based on serology and 7 on CISH). The median age of the non-HIV group and WLWH was 53 years and 49 years, respectively. There was no significant difference in age distribution ($p = 0.9681$).

According to the 5th edition of the WHO 2020 classification of female genital tumours, HPV-associated morphology was observed in all cases, both WLWH and non-HIV. There were no significant differences in the frequency of major types (usual *versus* mucinous) ($p = 0.6789$).

Regarding p16 expression, 12 cases in the WLWH group exhibited complete block positivity (score 3), while two cases showed partial staining (score 2). In the non-HIV group, 21 cases had complete block positivity,

Table 1 Patient characteristics in relation to HIV status

Patient characteristics	**Non-HIV women**		**WLWH**		***p* value**
Age, median	53		49		*P* = 0.9681
Age (range/quartiles)	32–78/42;66		38–80/41;67		
Histopathological type of EAC	**n**	**%**	**n**	**%**	
HPV associated types					*p* = 0.6789
Adenocarcinoma, usual type	22	84.6	11	78.6	
Adenocarcinoma, mucinous type	4	15.4	3	21.4	
subtype NOS[a]	*1*	*3.8*	*0*	*0.0*	
subtype intestinal	*2*	*7.7*	*3*	*21.4*	
Subtype signet ring cell	*1*	*3.8*	*0*	*0.0*	
Total	26	100.0	14	100.0	
p16 status	n	%	n	%	
Negative	1	3.8	0	0.0	*p* = 0.1187
Positive, partial	4	15.4	2	14.3	
Positive, complete	21	80.8	12	85.7	
Total	26	100.0	14	100.0	

[a] *NOS* not otherwise specified

Table 2 HPV genotyping. HPV genotyping (total (n), and within parentheses stepwise approach with Anyplex II first run / second run / in-house / FISH)

	Non-HIV Women		**WLWH**		
hrHPV	**n**	**%**	**n**	**%**	***p*-value**
16	5 (2 /3[a]/0/0)	19.2	2 (0/2/ 0/0)	14.2	*P* = 0.2792
18	16 (8/0/1/7[c])	61.6	6 (5/0/1/0)	42.9	
45	5 (3[b]/0/0/2)	19.2	6 (1/0/0/5)	42.9	
Total	26	100	14	100	

Including [a]2 cases with double-infections HPV31 and HPV69, respectively, [b]1 case with double-infection with HPV35, [c]All 7 cases with double-infection with HPV45

four had partial positivity (score 2), and one case was completely negative (score 0).

HPV was detected in all 40 cases (100%) using a stepwise approach as outlined in Fig. 1. The Anyplex II assay identified the virus in 24 cases, with an additional two cases detected by in-house PCR. The remaining 14 cases were initially screened using CISH, resulting in 13 positive results. All 14 of these cases underwent type-specific FISH, revealing various combinations of hrHPV.

The detailed results of the stepwise genotyping analysis are presented in Table 2.

HPV18 was the most prevalent type, followed by HPV45 and HPV16. Importantly, all cases, regardless of whether they were single or double infections, included at least one of the hrHPV16/18/45 genotypes.

As illustrated in Fig. 2, multiple infections were exclusively observed in tumours from non-HIV women. In one case, the HPV Anyplex II assay detected HPV16 co-infected with HPV31 and HPV69, as well as HPV45/35. Additionally, FISH analysis identified HPV 18/45 in seven cases. Consequently, multiple infections were significantly more common among non-HIV women (10/26 cases, $p = 0.0002$). Figure 3 presents micrographs of CISH- and FISH-positive cases.

Discussion

This study presents a cohort of EAC cases from an HIV-endemic setting. While both HPV-associated and HPV-independent forms of EAC are recognised [16] questions remain regarding the pathogenesis and primary prevention of EAC by vaccination in WLWH patients, particularly in sub-Saharan Africa, where screening and antiretroviral therapy access is limited.

Our cohort of 40 EAC cases represents a subset of a larger cohort of over 700 CC patients diagnosed at Maputo Central Hospital. This highlights the relatively low proportion of non-squamous cases (5%) [29]. The healthcare system limitations in LMICs hinder comparisons with true population-based data on HIV-positive and HIV-negative populations. However, previous studies [23] suggest an increased risk of EAC among WLWH, although less pronounced than in SCC. Our cohort, with 35% WLWH, aligns with this trend, given the national HIV prevalence of 15% among women aged > 15 years [37].

Data on HIV serology was available for only 18 of the 40 patients. To address this, we performed a supplementary HIV status assay using CISH [38, 39] on whole tissue sections [35, 36], enabling HIV status determination for

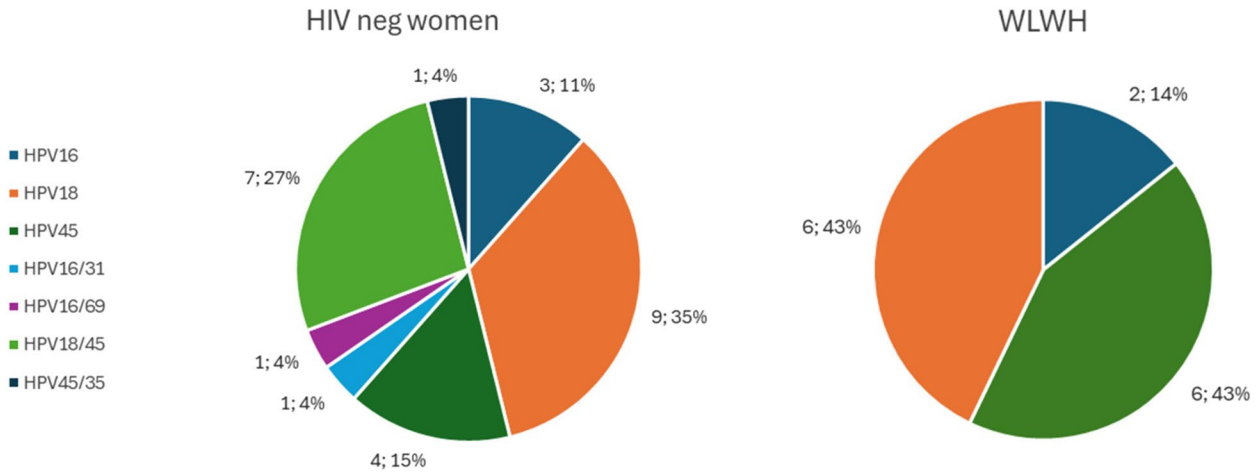

Fig. 2 Distribution of HPV multiple infections in non-HIV and WLWH

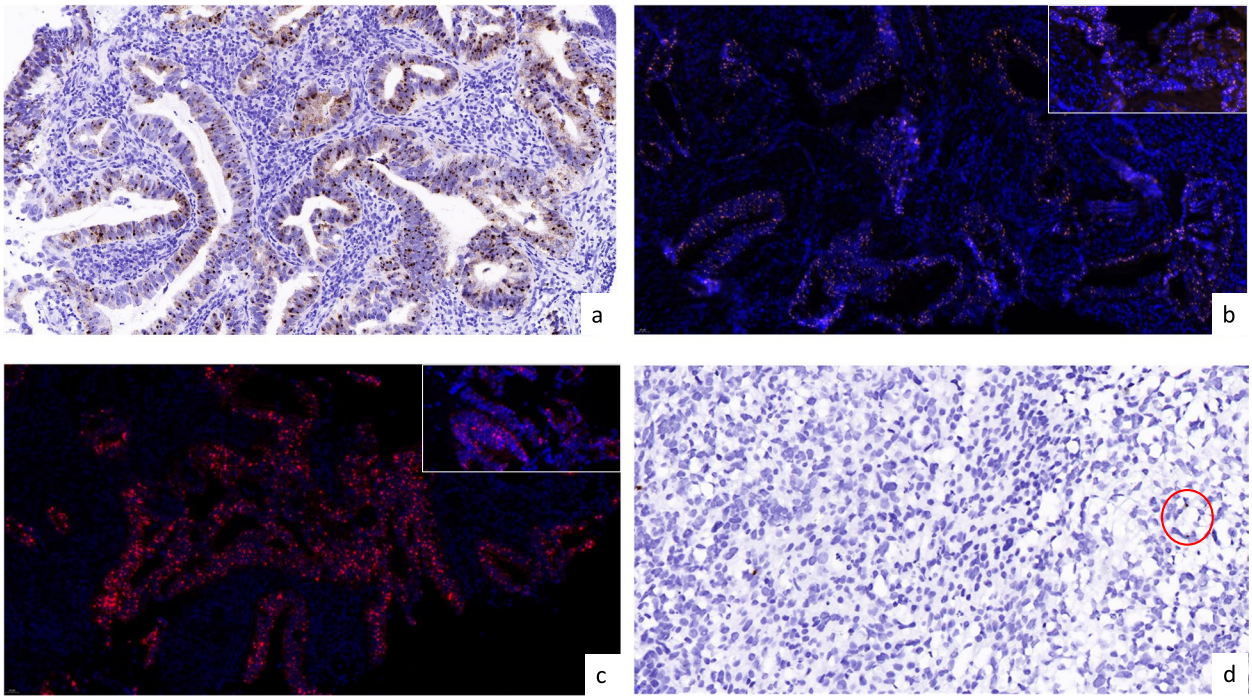

Fig. 3 Micrographs of in situ hybridisation. **a** CISH for 18 HPV Positive, brown dots in cytoplasm and nucleus (40x); **b** FISH HPV18 (40 × and insert 115.9x), **c** HPV45(40 × and insert 115.9x); **d** HIV positive Brown dots in immune cells -red circle (40x)

all cases. This method proved valuable in cases lacking HIV serological information.

Patient age in our study was comparable to previous reports [40], with no significant difference between WLWH and non-HIV groups. While age distributions vary across studies, some suggest a lower average age for WLWH (third and fourth decades) compared to non-HIV women (over 50 years) [24, 41]. Another study reported a high prevalence of newly diagnosed CC in women under 40, followed by the 40–60 age group, aligning somewhat with our findings [42]. Wu et al. reported a 10-year younger age for WLWH with CC in Uganda compared to HIV-negative women [43]. However, these studies did not especially analyse the EAC subgroup [42, 43].

HPV detection relied on a combination of methods, as DNA and mRNA detection can be challenging in formalin-fixed, paraffin-embedded (FFPE)

tissue. Combining methods and targeting both DNA and mRNA enhances detection sensitivity [44, 45]. PCR-based methods yielded negative or invalid results in some cases, potentially due to factors such as small core samples, low tumour cell numbers, degradation, or low HPV DNA copy numbers [46].

The cases that remained negative/invalid in the AnyplexII and in the in-house PCR, an assay with a general HPV probe from RNAscope® was performed on whole slide sections, which added further positive cases. The mRNA targeted CISh and FISH showed to be of great value in this cohort, as the 14 cases that were negative in PCR only one case remained negative in CISH [47, 48], furthermore all cases became positive using type specific FISH.

The WHO 2020 classification categorises EAC as HPV-associated or HPV-independent [16]. Our study found no HPV-independent tumours, which may reflect their rarity [15]. Furthermore, p16 expression, a surrogate marker for HPV, was observed in all but one case [44, 49–51].

HPV infection was detected in all EACs in the WLWH group as well as the non-HIV group, with HPV18 as the most prevalent type, followed by HPV45 and HPV16. Interestingly, all co-infections (HPV16/69, 16/31, 18/45, 45/35) occurred in the non-HIV group, aligning with reports of frequent co-infections in EAC [52, 53].

A key question in HIV-endemic settings is the vaccine efficacy of current quadrivalent and nonavalent vaccines compared to western data [54]. In all 40 cases, there was an infection covered by the quadrivalent vaccine. Our findings indicate that all cases were potentially preventable with the quadrivalent vaccine, considering cross-reactivity with HPV45 previously reported in literature [55].

Limitations of this study include its retrospective nature, potential selection bias due to hospital-based data, and limited clinical HIV serological information. However, the HIV status was obtained through the FFPE as addressed above in this section. The relatively small number of EAC cases restricts comparisons between HIV-positive and HIV-negative groups.

The number of EAC, is quite high when compared with other studies in LMIC environments [56, 57], the use of mRNA targeted ISH for HIV status determination, combined with multiple HPV detection methods, strengthens the study.

Mozambique included the HPV vaccination in the national vaccination calendar in November 2021 with the quadrivalent vaccine targeting HPV6/11/16 and 18 [58]. As HPV was detected in all cases of our cohort, our results are important as they show that WLWH will also benefit from the current vaccination, like non-HIV positive women.

During the coming years, further aspects of vaccination must also be considered. Presently the vaccines cover the most frequent hrHPV types, whether these will be eradicated [59] or non-vaccine-preventable HPV, termed clinical unmasking, will evolve in the vaccinated population [60] needs to be monitored, especially in the HIV endemic milieu. Furthermore, the potential therapeutical use of vaccines in the context of early disease might be a promising development [61], but once again needs to be addressed in the regional context of the HIV endemic situation.

Conclusions

Our findings contribute to understanding EAC aetiopathogenesis in HIV-endemic settings and highlight the potential impact of the current HPV vaccination. The detection of HPV in all cases emphasises the importance of vaccination programmes, especially considering the recent introduction of the quadrivalent HPV vaccine in Mozambique.

Abbreviations

CC	Cervical cancer
CISH	Chromogenic In Situ Hybridization
DNA	Deoxyribonucleic acid
EAC	Endocervical adenocarcinomas
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescent In Situ Hybridization
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
hrHPV	High-risk human papillomavirus
ISH	*InSitu* Hybridization
LMIC	Low- and middle-income countries
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
SCC	Squamous cell carcinomas
TMA	Tissue micro array
VIA	Visual Inspection with 3–5% acetic acid
WHO	World health organization
WLWH	Women living with HIV

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Authors' contributions

LL conceptualized and designed the study, selected cases for the study, performed data acquisition, revised morphological diagnosis, TMA construction, interpreted TMA immunohistochemistry, PCR, in situ hybridisation and drafted the manuscript. GLL interpreted molecular analysis of PCR and critically revised the manuscript. ST performed clinical data acquisition. CC conceptualized and designed the study, revised morphological diagnosis, interpreted TMA immunohistochemistry, ISH and critically revised the manuscript. SA conceptualized and designed the study and critically revised the manuscript. CK conceptualized and designed the study, performed data acquisition and

analysis, interpreted TMA immunohistochemistry, ISH critically revised the manuscript. All authors have read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this article. Further enquiries can be redirected to the corresponding author.

Declarations

Ethics approval and consent to participate
The study was approved by the National Bioethics Committee for Health (CNBS) board of Mozambique under registration number 114/CNBS/2019 and Swedish Ethical Review Authority under registration number 2023–01674-01–423571, according to the Helsinki Declaration in human subject research. The tissue material consists of formalin fixed paraffin embedded (FFPE) blocks of invasive cervical cancer samples from biopsy or surgery obtained and sent to the Pathology Department of Maputo Central Hospital for diagnostic purposes on 2017—2018. The tissue samples were strictly anonymized before inclusion in the present study. The informed written consent was waived from the study protocol by the CNBS. No further sampling, treatment or contact with the patients was needed. The new information received would not change any potential forthcoming treatment or outcome for the individual patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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