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Cervical Carcinoma in Kampala, Uganda and The Relationship With Human Immunodeficiency Virus and Human Papillomavirus Infections

Michael Odida

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CERVICAL CARCINOMA IN KAMPALA, UGANDA, AND THE RELATIONSHIP WITH HUMAN IMMUNODEFICIENCY VIRUS AND HUMAN PAPILLOMAVIRUS INFECTIONS

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Kampala and Stockholm 2010

ABSTRACT

Introduction: Cervical carcinoma is the commonest tumour in Ugandan women. However, very little is known in the Ugandan population about HPV types associated with invasive cervical cancer, the relative risk of cervical cancer associated with different HPV types, and whether the risk of cervical cancer associated with HPV infection is modified by HIV infection. Furthermore, the nature of HPV negative invasive cervical cancer, especially adenocarcinoma, is yet to be described and studied to find if the use of different types of cervical samples can influence the studies' results.

Objectives: To study HPV types associated with invasive cervical cancer. To estimate the relative risk for infections with different HPV types, and whether this is modified by HIV infection. To describe the nature of HPV negative cancer, especially adenocarcinoma, and study if the use of different invasive cervical cancer samples can influence the studies' results.

Subjects and Methods: We conducted studies using 186 archival cases of cervical carcinoma diagnosed between 1967 and 1980, 316 new incident cases of cervical cancer and exfoliated cervical cells from 314 control women from Uganda between September 2004 and December 2006. HPV testing was done using the SPF10 LiPA technology. In paper I, we assessed the prevalence and HPV types. For Article II, we used different antibodies to clarify the nature of HPV negative adenocarcinomas. In Article III, we estimated the odds ratio (OR) of cervical cancer for different HPV infections as well as for HIV infection. In Article IV, we compared HPV detection in formalin-fixed paraffin embedded (FFPE) and fresh samples.

Results: In Article I, Out of the 186 cases of confirmed invasive cervical cancer in the study paraffin blocks, 114 were positive for HPV DNA. Specific HPV genotypes were identifiable in 109 cases: HPV 16, 18, 31, 35, 39, 44, 45, 51, 52 and 70. These occurred as single infections in 105 cases (96.3%) and as multiple infections in 4 cases (3.7%). HPV 16 or 18 accounted for 80% (84/105) of cases with a single infection.
In Article II, Among the 13/25 HPV negative ADC samples, five were positive for CEA, suggesting endocervical origin, and three were vimentin positive (one had a mucinous endocervical histological pattern and two were ADC, not otherwise specified, most likely of endometrial origin). In Article III, we found a high relative risk of both squamous cell carcinoma and adenocarcinoma of the cervix with HPV 16 and 18, and moderate relative risk with HPV 45. Concurrent infection with HIV did not increase the relative risk with these three HPV types, but was associated with a statistically borderline increased relative risk of squamous cervical carcinoma only with low-risk types and multiple infections with HPV types.
Article IV showed comparable HPV detection in both fresh and FFPE tissue by the SPF10 LiPA method, with complete agreement for 59.7%, partial agreement 5.3% and complete disagreement 35.0%.

Conclusion: Most cervical carcinomas in Uganda are associated with high-risk HPV types, and concurrent infection with HIV has little impact. Both archival and fresh samples are suitable for HPV detection, and the use of antibodies may be useful in the characterization of HPV negative cervical adenocarcinomas.

Key words: HPV, Human Papillomaviruses, women, HIV, Uganda, invasive cervical cancer.

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Dedication

This thesis is dedicated to good health of all women

ABSTRACT

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Conclusion: Most cervical carcinomas in Uganda are associated with high-risk HPV types, and concurrent infection with HIV has little impact. Both archival and fresh samples are suitable for HPV detection, and the use of antibodies may be useful in the characterization of HPV negative cervical adenocarcinomas.

Key words: HPV, Human Papillomaviruses, women, HIV, Uganda, invasive cervical cancer.

LIST OF PUBLICATIONS

I. **Odida M**, de Sanjose S, Quint W, Bosch XF, Klaustermeier J, Weiderpass E. Human papillomavirus type distribution in invasive cervical cancer in Uganda. *BMC Infectious Diseases* 2008; 8:85.

II. **Odida M**, Lloveras B, Guimera N, Weiderpass E.
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Comparison of Human papillomavirus detection between freshly frozen tissue and paraffin embedded tissue of invasive cervical cancer.
Infectious Agents and Cancer 2010; 5:15.

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LIST OF ABBREVIATIONS

ADC	Adenocarcinoma
AIDS	Acquired immunedeficiency syndrome
bp	Base pairs
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CEA	Carcino-embryonic antigen
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
DB	Dot blot
DDL	Delft Diagnostic Laboratory
DNA	Deoxyribonucleic acid
E1	Early gene 1
E2	Early gene 2
E3	Early gene 3
E4	Early gene 4
E5	Early gene 5
E6	Early gene 6
E7	Early gene 7
EBV	Epstein Barr virus
ER	Eostrogen receptor
FDA	Food and drug administration
FFPE	Formalin-fixed paraffin embedded
GP	General primer
H&E	Haematoxylin and Eosin
HAART	Highly active antiretroviral therapy
hc	Hybrid capture
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	High-risk
HRP	Horseradish peroxidase
ICO	Institut Català d' Oncologia
IHC	Immunohistochemistry
ISH	Insitu hybridization
KCR	Kampala Cancer Registry
L1	Late gene 1
L2	Late gene 2
LCR	Long control region
LiPA	Line probe assay
LR	Low-risk
MHC	Major histocompatibility complex
OD	Optical density
OR	Odds ratio
P14	Tumour suppressor protein $p14^{ARF}$

P16	$p16^{INK4a}$ protein
P53	Nuclear phosphoprotein encoded by p53 gene
PCR	Polymerase chain reaction
PR	Progesterone receptor
pRB	Retinoblastoma protein
RIS HPV TT	Retrospective international study on HPV distribution among cases of invasive cervical cancer
SB	Southern blot
SCC	Squamous cell carcinoma
SIR	Standardized incidence rates
SPF	Short primer fragment
TMA	Tissue microarray
URR	Upper regulatory region
VIM	Vimentin

1 INTRODUCTION

In Uganda, cervical carcinoma is one of the commonest tumors affecting women. Data from the Kampala Cancer Registry (K.C.R) in the fifties showed the incidence was 12.6/100,000 [1]. Subsequent results for the period 1960 to 1997, showed the incidence increasing from 22 in the 1960s to 40/100,000 in the 1990s [2] and has now risen to 52.4 per 100,000 women [3]. Whether the increase could be partially attributed to the prevailing AIDS epidemic remains unclear. Earlier studies done in Uganda [4] and Tanzania [5] found no association between cervical cancer risk and Human immunodeficiency virus (HIV), while more recent studies from Uganda showed marginal increased relative risk of cervical cancer among women who are HIV positive [6, 7].

This apparent discrepancy is difficult to explain, but could be due to the lack of early detection and screening programs for cervical cancer, and the relatively short life expectancy of HIV positive women, in particular before the introduction or anti-retroviral drugs. Thus, it is plausible that a significant percentage of HIV-positive women could die before developing cervical carcinoma in many countries in Sub-Saharan Africa, including Uganda.

Despite the high incidence of cervical carcinoma in Uganda, not much is known about Human papillomavirus (HPV) types associated with it, although a number of studies have been carried out on different aspects of HPV and cervical cancer [4, 6-9]. It is therefore important to estimate the relative risk of cervical carcinoma with different HPV and the effect of HIV infection on the risk in the Ugandan population as well as to clarify the nature of HPV negative cervical cancers, especially adenocarcinoma. These are important background for the development and evaluation of future HPV vaccines.

This thesis is composed of 4 original scientific articles, namely:

Article I. HPV type distribution in invasive cervical cancer in Uganda.

Article II. The usefulness of immunohistochemistry in tissue microarrays of human papillomavirus negative adenocarcinoma of the uterine cervix.

Article III. HPV types, HIV and invasive cervical carcinoma risk in Kampala, Uganda: a case control study.

Article IV. Comparison of human papillomavirus (HPV) detection between fresh tissue and paraffin embedded blocks of invasive cervical cancer.

2 BACKGROUND

2.1 OVERVIEW OF HUMAN PAPILLOMAVIRUS (HPV)

HPV are DNA viruses belonging to the Papillomaviridae family. To date, more than 100 HPV types have been described, and many have their genomes partially or fully sequenced [10]. Despite their marked diversity, HPV share a common genetic organization. The circular DNA genomic structure is divided into three parts. The first part is known as the non-coding regulatory region, also termed the up-stream regulatory region (URR) or long control region (LCR), and it modulates viral replication and gene transcription. The second part is the HPV early genes (E1, E2, E4, E5, E6, and E7), which code for proteins involved in viral replication, viral transcription and regulation of infected cell proliferation. The last part is the late region composed of two genes (L1 and L2) that code for the minor and major capsid viral proteins.

HPV are divided into 16 genera, with the alpha- and beta-papillomaviruses being associated with human lesions. The alpha-papillomaviruses are associated with most ano-genital lesions [10]. HPV types that were initially detected from benign lesions were classified as low-risk types, while those detected from malignant lesions were designated as high- risk types.

2.2 HPV DETECTION METHODS

A number of methods have been developed to detect HPV, the most widely used being PCR-based protocols using primers that are directed against the highly conserved region of the L1 gene and which are able to detect many mucosal HPV types (Bernard et al., 1994). The GP5+/6+ system [11] utilizes 150 bp primers, while the MY09/11 primer [12] and primer derivatives use 450 bp primers

[13]. The primers for SPF10-PCR assay [14, 15] use primers which are 65 bp and are more specific compared to the others.

The other PCR-based methods are the hybrid capture systems. The Hybrid Capture 2 HPV DNA test (hc2) (Digene Corporation, Gaithersburg, MD), a microplate-based solution and solid phase hybridization assay for the detection of 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and five low-risk (6, 11, 42, 43, 44) HPVs, is at present the only FDA approved assay for the routine detection of HPV infections and the only commercially available HPV DNA assay with sufficient scientific data to support its performance in a clinical setting (2, 6, 7).

Other molecular methods include Southern blot (SB), dot blot (DB) and in situ hybridization (ISH). With the exception of ISH, which is popular among pathologists because of its direct visualisation effects, SB and DT have been mainly replaced by PCR methods.

2.3 HPV AND CERVICAL CARCINOMA

Clinical and epidemiological studies have clearly established that infections by certain human papillomaviruses (HPVs) types, so-called high-risk types, are intimately linked to cervical cancer development. Several types of HPVs, especially types 16 and 18, are recognized as the main causes of cervical cancer and its precursor lesions [16]. These two viral types were found in most cases of invasive cervical cancer from 22 countries around the world [17].

From earlier studies, HPV were classified into high-risk (HR type) or low-risk (LR type) based on their isolation from malignant or benign lesions [18, 19] ,while taxonomic classification is based on DNA sequences of the L genes [10]. Using epidemiologic approaches, Muñoz et al. [20] classified HPV into high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), probably

high-risk types (26, 53 and 66), low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and CP6108) and lastly some as undetermined risk types (34, 57 and 83). The risk of cervical carcinoma associated with HPV varies in different studies. Franceschi et al. [21] assessed the risk of cervical carcinoma in India and found an OR of 497.9 (95% CI: 67.7-999) for any positive HPV. Castellsagué et al. [22] assessed the risk of cervical adenocarcinoma and found increased relative risk for any HPV positive (OR 81.27, 95% CI: 42.04-571.1), HPV 16 (OR 164.12, 95% CI: 76.09-354) and HPV 18 (OR 410, 95% CI: 167.44-∞). In a recent study from Mexico, Illades-Aguiar et al. [23] also found high ORs, varying from 34.9 (95% CI: 2.8-∞) for low-risk HPV to 804.4 (95% CI: 71.1-∞) for HPV 18.

There are relatively few studies that have assessed the risk of cervical carcinoma in relation to HPV and HIV. Hawes et al. [24] found increased relative risk of cervical carcinoma for high- risk HPV types in association with HIV infection, with ORs of 2.2 (95% CI: 1.0-4.8) for HIV1, 6.0 (95% CI: 2.1-17.1) for HIV2 and 8.0 (95% CI: 2.0-31.5) for HIV1 and 2.

A number of HPV types have been identified in cervical carcinoma from different studies, with HPV 16 and 18 occurring in more than 50% of the cases, with geographical variations. A large worldwide study was done by Bosch et al. in the early nineties [17]. Over 1000 cases of invasive cervical carcinoma from 22 countries were analyzed. HPV DNA was detected in 93% of the cases. Overall, HPV 16 was predominant in all countries except Indonesia, where HPV 18 was predominant. In a study from China, Chen et al. [25] analyzed 664 cases of invasive cervical carcinoma (630 SCC, 34 ADC) and 569 CIN 2/3 from seven regions of China. HPV was detected in 97.6% of invasive cancer, with HPV 16 being the most common.

In a pooled analysis of 10,058 cases (8550 SCC, 1508 ADC) [26], the most common HPV types in ICC were, in order of decreasing prevalence, HPV 16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 6, 51,

68, 39, 82, 73, 66 and 70. In SCC, HPV 16 was the predominant type (46-63%), while in ADC HPV 18 was the predominant type in every region (37-41%). In studies restricted to ADC only, the HPV prevalence was high. Castellsagué et al. [22] found HPV 16 and 18 were present in 82% of cases, while An et al. [27] found 78% of ADC had HPV 16 or 18.

2.4 TYPES OF CERVICAL CARCINOMA

Cervical carcinoma comprises of squamous cell carcinoma, adenocarcinoma and other types. Squamous cell carcinoma is the commonest type, accounting for more than 70% of cases. HPV 16 tends to predominate in squamous cell carcinoma, while HPV 18 types are usually found in adenocarcinomas. Though these two types are associated with the two histological types, other types of HPV have also been identified in these histological types. While the diagnosis of SCC is usually straightforward, the nature of adenocarcinomas in the cervix may be difficult to ascertain whether of cervical or endometrial origin.

There are also some rare types of cervical carcinoma, namely one resembling lympho-epithelioma [28, 29]. Etiologically, some are thought to be related to EBV [30], with some being negative for HPV [31]. They are more common in Asian women than Caucasians [32]. Other unusual forms of cervical carcinoma are papillary squamous cell carcinoma [33-35]. Unusual types of adenocarcinoma include glassy cell carcinoma [36], clear cell, serous, and mesonephric carcinomas [37].

2.5 MECHANISMS OF HPV CARCINOGENESIS

The roles of HPV in cervical carcinoma are thought to be through interference with p53 and retinoblastoma gene product. The E6-p53 interaction results in the accelerated degradation of p53 in vitro via the ubiquitin-dependent proteolysis system [38, 39], while the E7 complexes with pRB [38], the affinity of HR HPV being ten times that of other HPV [40]. Use of the E7-E6 fusion proteins of HPV 6 and HPV 11 have also been shown to promote the degradation of the retinoblastoma protein [41], indicating that the property to target associated proteins for degradation is shared by the anogenital specific HPV E6 proteins. In addition to interference with p53 and pRB pathways, the E6 and E7 HPV proteins induce chromosomal alterations [42].

The inhibition of the retinoblastoma gene leads to accumulation of p16 [43] and this has been used as a surrogate marker of HPV infection [44] and also as a marker of *E7* gene activity [45].

2.6 HPV NEGATIVE CERVICAL CARCINOMA

Although many cases of cervical carcinomas are associated with HPV, there are cases where HPV have not been detected. Liebrich et al. [46] described four cases of HPV negative cervical carcinoma in 14 to 20 years old cases out of 5,878 cervical carcinoma cases. They also found 14 cases in a literature search. Pirog et al. [37] earlier noted that some rare types of endocervical adenocarcinoma were negative for HPV DNA. Kanao et al have shown that over-expression of p14(ARF) and p16(INK4A) was strongly associated with HPV positive cervical carcinoma, while reduced expression of p14 and p16 correlated with HPV negative cervical cancer [47]. Abnormalities of G1 pathway may also be one important mechanism of carcinogenesis of HPV negative cervical carcinoma [48].

2.7 MECHANISMS OF EVASION OF IMMUNE SYSTEM BY HPV

HPV appears able to evade the immune system, although the exact mechanisms are not clear. A number of mechanisms for evasion of the immune system have been suggested. Tindle [49] suggested this could be due to avoidance of immune response by HPV. Other ways include inhibition of MHC by E6 and E7 proteins [50, 51], manipulation of Langerhan cells by minor capsid proteins [52], inhibition of transcription factors [51] and subverting the immune response [49, 53]. It has also been experimentally shown that HPV 16 interferes with innate immunity by affecting TLR9 expression and function [54]. In addition to evasion of the immune system, HPV may persist in the cervix because it could be an immune privilege site [55].

2.8 COFACTORS IN CERVICAL CARCINOGENESIS

It is well known that HPV is essential, but not sufficient, to cause cervical carcinoma. Other cofactors are necessary. In addition to factors associated with lifestyle, a number of cofactors have been studied, namely, Clamydiae trachomatis [56, 57], hormonal factors [58-60] and immune deficient states [61-64] and many others. Immune deficient states have aroused much attention by finding increased relative risk of tumours in transplant recipients [65-70].

2.8.1 Cofactors associated with HPV acquisition

From earlier observations, it was noted that cervical carcinoma was very rare in nuns and virgins, but much more common in prostitutes (summarized by zur Hausen, 2009) [71]. This could be explained by acquisition of the causative agent through sexual intercourse. These were subsequently confirmed by additional studies who found increased relative risk of cervical

carcinoma in association with early age at first sexual contact, multiple sexual partners, women whose husband has multiple sexual partners, educational status, etc. Studies have shown that two out of three people who have sexual intercourse with a HPV positive person acquire the infection [72].

2.8.2 Cofactors associated with HPV persistence

A number of studies have demonstrated increased relative risk of cervical carcinoma in women exposed to certain situations. These include, for example, hormonal contraceptives, smoking and immune suppression due to a number of agents.

2.9 HORMONES AND CERVICAL CARCINOMA RISK

Sex hormones have been implicated in HPV associated carcinogenesis [73]. In a pooled analysis on reproductive factors, oral contraceptive use and HPV, it was suggested that these could be involved in the transition from HPV infection to neoplastic cervical lesions [74] and not HPV prevalence.

2.10 CERVICAL CARCINOMAS IN ASSOCIATION WITH IMPAIRED IMMUNE SYSTEM

2.10.1 Risks of cervical carcinoma in transplants recipients

A number of studies have assessed the risk of cervical carcinoma in organ transplant recipients [67]. Most of the studies found statistically significant increased risk of cervical carcinoma as shown in the tables below. A surprising result was noted by Adami et al. [65] who found a non-statistically increased risk of cervical carcinoma, but noted a much higher risk for vulva and vagina carcinoma.

Table 2.1 Relative Risk of cervical carcinoma in transplant recipients.

Author [ref]	Year	No cases	Relative Risk	95% CI
Adami et al. [65]	2003	5	2.0	0.7-4.7
Busnach et al. [66]	2006	3	3.3	0.7-9.7
Kessler et al. [67]	2006	6	25.28	9.27-55.02
Meeuwis et al. [68]	2009	1	3.2	-
Schulz et al. [75]	2009	3 studies	2.13	1.37-3.30
Serraino et al. [69]	2006	3	3.3	0.7-9.7
Srisawat et al. [70]	2008	2	789.83	88.76-2851.16

2.10.2 Risks of cervical carcinoma in HIV infection

With the advent of the AIDS epidemic, an increased incidence of tumours, especially Kaposi's sarcoma, was observed as a consequence of immunodeficiency [76]. Extending this approach, a number of investigators assessed the possible role of HIV in cervical carcinoma. Some studies from developed countries have found increased relative risk of cervical carcinoma in association with HIV as shown in the tables below.

Table 2.2 Relative Risk (OR) of cervical carcinoma associated with HIV.

Author [ref]	Year	No cases	Relative Risk (OR)	95% CI
Biggar et al. [77]	2007		1.41	0.81-2.46
Busnach et al. [66]	2006	22	14.6	9.1-22.1
Frish et al. [78] pre AIDS	2000	44	5.4	3.9-7.2
Frish et al. [78] post AIDS	2000	15	5.1	2.9-8.5

Serraino et al. [69]	2007	22	14.6	9.1-22

Table 2.3 Standardized Incidence Rates (SIR) of cervical carcinoma associated with HIV.

Author [ref]	**Year**	**No cases**	**Relative Risk (SIR)**	**95% CI**
Allardice et al. [79]	2003	1	1.7	0.04-9.26
Chaturvedi et al. [80]	2009	192	5.6	4.8-6.5
Clifford et al. [81]	2005	6	8.0	2.9-17.4
Dal Maso et al. [82]	2003	18	21.8	12.9-34.6
Engels et al. [83]	2008	28	2.9	1.9-4.2
Engels et al. [84] (1)	2006	10	7.7	3.7-14.1
Engels et al. [84] (2)	2006	34	4.2	1.8-3.1
Engels et al. [84] (3)	2006	30	5.3	3.6-7.6
Newnham et al. [85]	2005	3	1.0	0.2-2.9
Schulz et al. [75]	2009	6 studies	5.82	2.96-11.3
Serraino et al. [69]	2007	22	14.6	9.1-22
Srisawat et al. [70]	2008	2	789.83	88.76-2851

Studies from Africa have not demonstrated a similar marked increased of relative risk of cervical carcinoma as a result of HIV, as detailed in the table below [4-7, 86, 87], although the risk of pre-neoplastic lesions have been reported to be high in some studies [88-91].

Table 2.4 Relative Risk (OR) of cervical carcinoma associated with HIV from some African studies.

Author [ref]	Year	No cases	Relative Risk(OR)	95% CI
Holmes et al. [86] (HIV1/2)	2009	149	6.5	2.1-19.8
Holmes et al. [86] (HIV1)	2009	149	8.2	1.3-50.0
Holmes et al. [86] (HIV2)	2009	149	5.3	1.3-21.4
Kahesa et al. [87]	2008	138	2.9	1.4-5.9
Newton et al. [6]	2001	65	1.6	0.7-3.6
Schmauz et al. [4]	1989	34	0.23	0.05-0.96
Sekirime et al. [7]	2007	116	1.26	0.45-3.49
Sitas et al. [92]	2000	1323	1.6	1.1-2.3
Stein et al. [93]	2008	1586	1.6	1.3-2.0
Ter Muelen et al. [5]	1991	50	0.96, p-value 0.03	Not given

2.11 HPV PERSISTENCE AND HIV

HPV persistence as a consequence of HIV infection could be attributed to a number of mechanisms. HIV has been known to turn the cervical milieu to a more favourable for HPV persistence [94]. Persistence could also be due to dysregulation of local cervical humoral immunity as a consequence of HIV infection [95].

This apparent discrepancy is difficult to explain, but a lack of early detection and screening programs, and the relatively short life expectancy are possible explanations. Thus, a significant

percentage of cervical cancers in HIV-positive women in Sub-Saharan Africa could not have been detected before their deaths.

2.12 NATURAL HISTORY OF HPV INFECTION

HPV-induced cervical cancer is a multi-step process. HPV DNA in the cervix uteri is frequently detected in women at all ages, most frequently among young women soon after the start of having sexual intercourse. However, in the majority of the women, HPV infection does not lead to a clinical manifestation and is cleared by the host immune system in a relatively short time (6-12 months) [72, 96, 97]. In a small percentage, HPV infection becomes persistent and induces the development of low- and/or high-grade cervical intraepithelial neoplasias (CIN), which can still regress or progress to an invasive cervical carcinoma after a period of latency. These lesions are usually localized, although why they remain sharply demarcated is not clearly understood [98].
Factors important for the progression to cervical carcinoma are the virulent properties of the virus and the ability to evade the immune system.

2.13 STUDIES ON CERVICAL CARCINOMA DONE IN UGANDA

One of the first studies on HIV and cervical carcinoma was done at the beginning of the AIDS epidemic by Schmauz et al. [4]. However, the sample size was too small (50 controls and 34 cancer patients only) to reach any definite conclusions. Two other studies done later found no association of HIV and cervical carcinoma [6, 7]. These two studies did not assess the possible role of HPV. A study on the risk of cervical carcinoma as a consequence of HPV was done by Newton et al based on HPV serology only [8]. The relative risk of cervical cancer was associated with a higher level of

HPV antibodies. Some recent studies have focused on the prevalence of HPV types in women without cervical carcinoma. One study only included adolescents [99] , while the other two studies were in women up to the age of 49 [100] and 51 [101] years respectively. These studies found a high prevalence of HPV in the women. An earlier study on HPV variants in genital cancers [102], found HPV 16 Af1 sequences in 100% of tumour tissue and in 6.25% of scrapes. The authors concluded that progression to cancer is mainly associated with the HPV 16 Af1 variant.

From the foregoing, it becomes apparent that there are some gaps in knowledge about HPV and subsequent events in cervical carcinoma. For example what are the types of HPV in cervical carcinomas, and how does the presence of HPV DNA affect the biological behaviour of tumours. Does immunosuppression increase the risk of HPV associated cervical carcinoma?

Apart from the studies from this thesis, many previous studies have not adequately addressed these gaps. The characterization of the HPV spectrum and the interaction of HPV with HIV in the Ugandan population would provide important background information for the development and evaluation of future HPV vaccines.

3 AIMS OF THE THESIS

This thesis addresses some aspects of invasive cervical carcinoma in Uganda. The main aims are:

1. To ascertain the types of HPV distribution in invasive cervical cancer in Uganda.

2. To ascertain the usefulness of immunohistochemistry profiles in tissue microarrays in archived samples of adenocarcinoma of the uterine cervix that had been tested for HPV DNA, and in particular to assess if it would be possible to define the tissue of origin of HPV negative adenocarcinoma samples.

3. To estimate the relative risk of cervical carcinoma in association with different HPV types and HIV in Kampala, Uganda.

4. To compare HPV detection in fresh and paraffin embedded cervical carcinoma specimens.

4 MATERIALS AND METHODS

4.1 STUDY SITE

4.1.1 Studies reported in papers I and II

The study was conducted at the Department of Pathology, Makerere University. This department receives biopsy specimens from most hospitals in Uganda. Until the early nineties, the department was the only laboratory offering diagnostic histopathology services in Uganda. 186 cases of histologically diagnosed cervical carcinomas were obtained from the archives. The tissues were re-embedded in fresh paraffin wax and sectioned at four micrometer thickness. The first sections were stained with heamatoxylin and eosin (H&E) to confirm the diagnosis and also suitability of sample for HPV testing. Subsequent sections were the put in proteinase K for polymerase chain reaction. A third section was obtained to re-confirm the presence of tumour in the sections taken for PCR. For paper II, 26 cases of adenocarcinoma with known HPV status were stained with a panel of antibodies, namely, p16, eostrogen, progesterone, vimentin and carcinoembryonic antigen.

4.1.2 Studies reported in papers III and IV

The study was conducted in Mulago Hospital, which is the national referral and teaching hospital for the Faculty of Medicine, Makerere University, Kampala, Uganda. The Gynaecological Unit has three wards on the fifth floor viz. 5A, 5B and 5C. Each ward has 40 beds for gynaecological patients. The Unit has an emergency gynaecological section on ward 5A and runs three gynaecological clinics per week. Gynaecological cases are admitted through the gynaecological clinics or the emergency section on ward 5A. On average, each clinic attends to about 150 patients and admits about 10 cases with cervical cancer per month. Patients attending the Gynaecological clinics or emergency section are residents of Kampala City with a

population of about 1,000,000 and the surrounding areas. A few cases are referred by medical practitioners, although most come directly.

4.2 STUDY SUBJECTS

Patients eligible for the study included all women consecutively diagnosed with incident invasive cervical carcinoma who present to Mulago Hospital, Kampala, and gave their consent to participate in the study during the period September 2004 and December 2006. Cases were recruited from among women admitted to the gynaecological unit, and had a histologically confirmed diagnosis of cervical cancer.

Inclusion criteria:

- incident cases who have not yet undergone primary treatment for cervical cancer
- resident of Uganda for at least two years
- aged 18 to 74 years
- ability to give informed consent
- willing to provide samples

Exclusion criteria:

- incident cases who had already undergone primary treatment for cervical cancer
- resident of Uganda for less than two years
- aged less than 18 or more than 74 years
- inability to give informed consent
- not willing to give samples
- those terminally ill

Patients were excluded if tissue and blood samples could not be collected because they were in poor health or for any other reason that might interfere with established patterns of patient care.

A log form was kept throughout the study period. All potential participants in the study were recorded, and for non-participants, the reasons were also be recorded (e.g., refusal, terminal patient, etc.).

The goal of enrolling all consecutive patients was aimed at reducing bias that would occur if any criteria were used to select cases, for example stage, duration of symptoms, tribe, etc.
Control women were recruited from among women accompanying or visiting cervical cancer patients.

Inclusion criteria:

- resident of Uganda for at least two years
- aged 18 to 74 years
- ability to give informed consent
- willing to provide samples

Exclusion criteria:

- resident of Uganda for less than two years
- aged less than 18 or more than 74 years
- inability to give informed consent
- not willing to provide samples

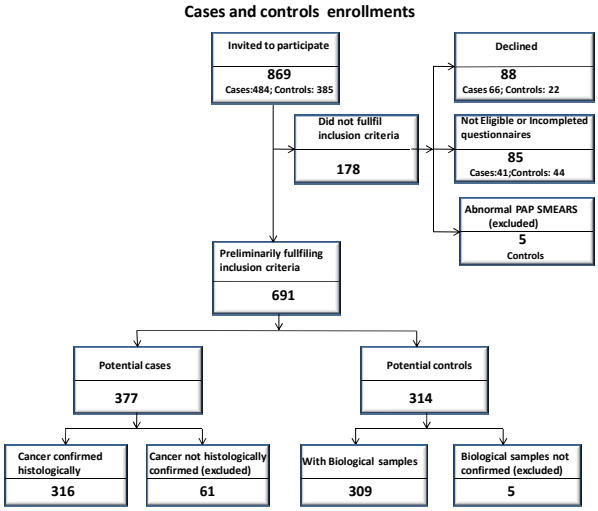
In Uganda, all hospitalised patients have with them one or more accompanying persons who are responsible for preparing food and taking care of the basic needs of the patients. These accompanying persons are in general women members of the same family or clan, having or not direct family blood links with the patient. We first invited women who were closest in age to the case patient. Since we did not intend to do individual matching of cases and controls, but only frequency matching by 5-years age groups, more than one control woman

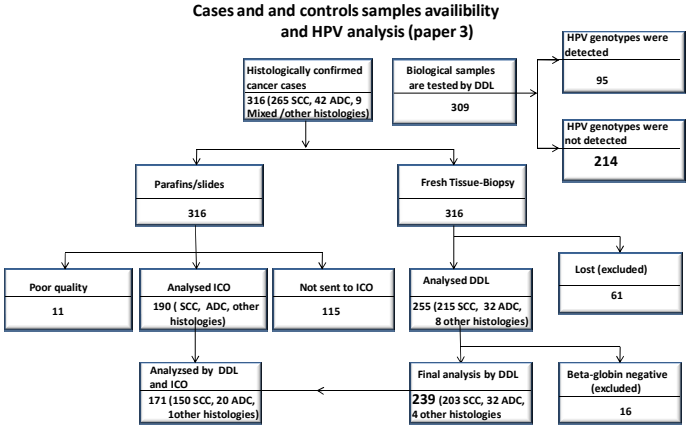
accompanying a cancer case were sometimes included in the study. This allowed for compensation for refusals or unavailability of suitable accompanying women among certain cancer patients.

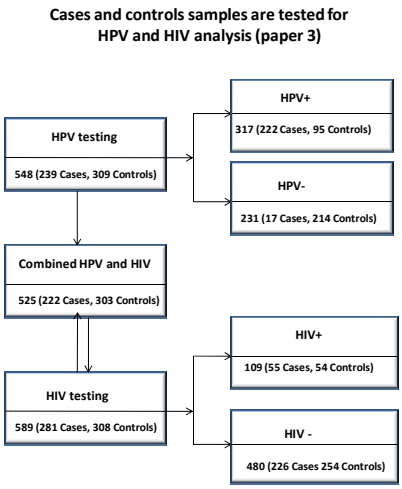
The motivation of enrolling visitors to the cancer patient was to assure the inclusion of control women who live in the same areas of the country as cancer cases. This was necessary, as the prevalence of HIV in Uganda varies broadly from one area to another.

Control women were offered visual inspection of the cervix uteri and a Pap smear test. Women diagnosed with pre-malignant and malignant cervical abnormalities were referred for standard treatment at the Mulago Hospital. Results from the Pap smear test were made available quickly enough to allow women to get the Pap smear result before leaving the hospital (i.e., during the case patient period of hospitalization). The gynaecologist examining control women offered appropriate antibiotic treatment to those with a syndromic diagnosis of cervical and vaginal infections.

The study subjects for articles IV consisted only of cancer cases which were analyzed at both DDL and ICO. The recruitment details are shown in the flow chart below.


Cases and controls enrollments
Invited to participate
869
Cases:484; Controls: 385
Declined
88
Cases 66; Controls: 22
Did not fullfil inclusion criteria
178
Not Eligible or Incompleted questionnaires
85
Cases:41;Controls: 44
Abnormal PAP SMEARS (excluded)
5
Controls
Preliminarily fullfiling inclusion criteria
691
Potential cases
377
Potential controls
314
Cancer confirmed histologically
316
Cancer not histologically confirmed (excluded)
61
With Biological samples
309
Biological samples not confirmed (excluded)
5


Cases and and controls samples availibility and HPV analysis (paper 3)
Histologically confirmed cancer cases
316 (265 SCC, 42 ADC, 9 Mixed /other histologies)
Biological samples are tested by DDL
309
HPV genotypes were detected
95
HPV genotypes were not detected
214
Parafins/slides
316
Fresh Tissue-Biopsy
316
Poor quality
11
Analysed ICO
190 (SCC, ADC, other histologies)
Not sent to ICO
115
Analysed DDL
255 (215 SCC, 32 ADC, 8 other histologies)
Lost (excluded)
61
Analyzsed by DDL and ICO
171 (150 SCC, 20 ADC, 1other histologies)
Final analysis by DDL
239 (203 SCC, 32 ADC, 4 other histologies
Beta-globin negative (excluded)
16



4.3 DATA COLLECTION PROCEDURES

The data were collected using questionnaires and laboratory tests. The details of how the cases and controls were enrolled and the number tested for HPV are shown in the flow chart above.

4.3.1 Questionnaires

All the study subjects were administered standard questionnaires asking for information about social status, reproductive history parity, age at first child birth, number of abortions, lifestyle, alcohol consumption, smoking, sexual practices, contraceptive use, age at first intercourse, number of lifetime sexual partners, history of sexually transmitted diseases and their frequencies and demographic details age, educational status, marital status and economic status including sources of income.

4.3.2 Collection of biological samples

Tumour samples were obtained from patients with cervical carcinoma, and exfoliated cervical cell samples were obtained from control women using cervical brushes.

Among women with invasive cervical carcinoma, biopsies of the cervical lesions were collected under general anesthesia. Samples were immediately stored into sterile specimen tubes and transported in a flask containing ice to the pathology laboratory. There the specimen was divided into two parts: one part was fixed in 10% buffered formalin (for 12 to 24 hours) for histological processing and diagnosis, and the other half was kept in a Nuc tube, labeled and stored at -80^{O}C until shipped for HPV analysis. In case the tissue was deemed to be very small, the whole tissue was processed for histological diagnosis. The biopsy samples were processed using the automatic tissue processor. They were paraffin embedded, sectioned at 4 µm thickness and stained with Haematoxylin and Eosin.

Among control women, exfoliated cervical cells were collected before any treatment with two Cervex brushes. The first brush was rinsed in PreservCyt solution (ThinPrep, Cytyc Corporation) according to the manufacturer's instructions and kept at room temperature until shipped to the laboratory for HPV testing. The second brush was used to make a smear and stained with Pap stain; smears were classified as normal or abnormal.

4.3.3 Laboratory tests

4.3.3.1 T cell counts

This was done using flow cytometry with the Bectondickinson automatic FACS count instrument. In addition to CD4 counts, it also gives the CD8 count and CD4:CD8 ratio. The analysis was done at Ebenezer Clinical Laboratory in Kampala (South African National Accreditation System Laboratory No. M0221, ISO 15189, International Organization for Standardization).

4.3.3.2 HIV test

The analysis was done at Ebenezer Clinical Laboratory in Kampala. HIV testing was offered to cases and controls. In case a woman manifested interest in knowing the result of the HIV test, pre- and post-test counseling were offered, according to a consolidate methodology available at the Mulago Hospital. Routine HIV testing and counseling has been found to be highly acceptable in Ugandan hospitals [103].

This was done using rapid tests as recommended by Downing et al. [104]. Briefly, blood was initially tested by the Capillus method (Capillus HIV-1/HIV-2 (CP) Trinity Biotech, Galway, Ireland). Negative results were reported as negative, and no other confirmatory tests were done. For positive results, a confirmatory test using the Serocard (Trinity Biotech, Galway, Ireland) was performed.

When the results from the Serocard were positive, the results were reported as positive. However, if the results from the Serocard were negative, a tiebreaker test using the Multispot test (Bio-Rad Laboratories) was done, and the result reported as negative or positive. The tests were done according to the manufacturer's instructions. HIV was assessed as positive (exposed) or negative (not exposed).

4.3.3.3 HPV tests

All paraffin embedded samples were stored and transferred to the HPV testing laboratory at ambient temperature. The fresh specimens were stored at -80°C and transferred to the HPV laboratory using dry ice.

Proteinase K (Sigma) digestion for 16 hours at 56°C temperature was used to obtain a tissue lysate containing DNA from the paraffin inner 5 μm sections. SPF10 PCR was performed using 10 μl of a 1:10 dilution with water of the tissue digest in a final reaction volume of 50 μl [14]. The amplified PCR products were tested using a probe hybridization with a cocktail of conservative probes recognizing at least 54 mucosal HPV genotypes in a microtitre plate format for the detection of HPV DNA. Optical densities (OD450) were read on a microtitre plate

reader. HPV DNA positive samples were subsequently analysed by HPV SPF10-LIPA25 (version 1: produced at Labo Biomedical Products, Rijswijk, The Netherlands) [14], a reverse hybridization technique that detects 25 high-risk and low-risk HPV types (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, 74). The sequence variation within the SPF10 primers allows the recognition of these different HPV genotypes, except for the types 68 and 73, as their interprimer regions are identical and cannot be distinguished on this test. After PCR, 10 μl of the amplimers was used to perform reverse hybridization for HPV genotype identification. The positive hybridization on the strips is visualized as a purple band by means of a precipitating colour substrate on the probe site. Specimens that were HPV/DNA positive but did not hybridize with any of the 25 probes were coded as HPV type X (unknown type). Amplification of a fragment of the human β-globin gene was performed in all HPV/DNA negative samples to assess DNA quality. HPV type specific distributions were calculated among HPV positive women. All SPF10-LIPA25 PCR detection and typing was performed at the facilities of DDL Diagnostic Laboratories (DDL, Voorburg, Netherlands) and at Institut Català d'Oncologia (ICO, Barcelona).

4.3.3.4 Tissue Microarrays

Tissue Microarrays (TMA): Three punches (gauge 1mm) of pre-existing paraffin embedded tissues were obtained from each block and then re-embedded in an arrayed master block using the manual tissue microarrayer (Beecher Instruments, Silver Spring MD). The punch specimens were performed in the most representative areas of the tumours, discarding necrosis or artifacts. Four micron paraffin sections were cut from the TMA blocks and deparaffinised through alcohols and xylene before immunostaining.

4.3.3.5 Immunohistochemistry

Immunohistochemistry (IHC): Monoclonal antibodies included: CEA (clone B0194-11M-P, BioGenex), p16 ^{INK4a}, p16 (clone JC8, Biocare Medical), Vimentin (clone V9, Dako), estrogen receptor alpha, ER (clone 1D5, Dako), progesterone receptor, PR (clone PgR 636,

Dako). The tissue sections were pre-treated for antigen retrieval, and the Dako Autostainer universal staining system was used with the EnVision+ Dual Link System-HRP, a two-step IHC staining technique. This system is based on an HRP labelled polymer, which is conjugated with secondary antibodies.

The final reaction was done with diaminobenzydine and slides were counterstained with hematoxylin. The immunohistochemical stains were interpreted by one of the authors (BL) and the results were reported as positive or negative using the following criteria for each antibody. ER and PR were considered positive when more than 10% of the tumour cells displayed nuclear positive staining. p16 was considered positive/overexpressed if more than 75% of the tumour cells showed cytoplasmic and/or nuclear staining. CEA was considered as positive if more than 10% of the neoplastic cells showed cytoplasmic positive staining, and vimentin was considered positive only when more than 20% of the tumour cells displayed cytoplasmic staining. The cutoff points for most of the antibodies were those used for routine diagnosis at Barcelona, while the cutoff point of 75% for p16 was chosen because this cutoff point is both sensitive and specific for HPV related adenocarcinoma [105].

5 STATISTICAL ANALYSIS

5.1 ARTICLE I

Chi square statistics was used to detect differences categorical data.

5.2 ARTICLE II

The chi-square test was used to determine significant differences between HPV positive and HPV negative adenocarcinomas and expression of, p16, CEA, ER, PR and vimentin.

5.3 ARTICLE III

The Odds ratios (OR) and associated 95% confidence intervals (CI) were calculated using logistic regression models. We fitted age-adjusted models for each HPV type or groups of HPV types, as well as models also adjusting for HIV (positive or negative) and CD4 counts reduction per 100 cells, as a linear continuous variable. For cases where it was not possible to estimate the lower or upper limit of 95% CI, only one limit was estimated [106].

5.4 ARTICLE IV

The Kappa statistics and McNemar test were used to test the statistical significance of HPV detection between fresh samples and paraffin blocks, while the Chi square test was used to test the statistical significance of HPV detection between SCC and ADC in both fresh and paraffin blocks. Where appropriate, 95% confidence intervals were computed and the level of the statistical significance test was 0.05.

6 ETHICAL ISSUES

Permission to carry out research was obtained from the Research and Ethical Committee of the Faculty of Medicine and the Uganda National Council of Science and Technology. For studies 3 and 4, written informed consents were obtained from all participants.

The potential participant was informed about the study in the local language. She was explicitly given the opportunity to ask questions and discuss in more detail any concerns she may have had. If she accepted, she was then asked to sign a written, informed consent appropriately translated and read to her in the local language, accepting the objective, procedures, and constraints and benefits of the study.

Hospitalized patients are not routinely tested for HIV in Uganda, basically due to lack of resources for testing and treatment. Thus, the HIV testing and CD4 counting were done for study purposes only. During the pre HIV testing counseling, the women were told about causation and complications of AIDS. During the post-counseling sessions, the women were given explanations on how to live safely, and the availability of treatment options for those who were positive. In cases deemed necessary, referral to relevant health care providers were done for control women. Therefore, case patients and control women were asked in a private section with a specialized HIV counselor if they:

a) agreed to donate blood sample for study purposes,
b) agreed to having the blood tested for HIV,
c) if yes, if they wanted to know the results or not,
d) women willing to know the results of their HIV test had an appointment booked in advance with the HIV counselor working in the study team. The date was booked while the case patient was hospitalized, thus allowing both the case patients and control women access to information without extra trips to the hospital.

Results from the Pap smear test were made available quickly enough to allow the women to get the results before leaving the hospital (i.e. during the case patient period of hospitalization). All control women diagnosed with pre-malignant and malignant cervical abnormalities were referred for standard treatment at the Mulago Hospital.

Strict confidentiality was followed at all times, and no information was made public. All specimens were labeled with unique identification numbers and the corresponding names and document kept by the investigator in a locked cabinet.

7 SUMMARY AND DISCUSSION OF THE STUDIES' RESULTS

7.1 PAPER I

Of the 191 FFPE tissue blocks, each corresponding to a case of cervical cancer, five were excluded due to extensive necrosis of the samples on histological review, leaving 186 cases for HPV analysis. 146 were SCC, 35 were ADC, three were adenosquamous carcinoma, and two were undifferentiated carcinoma. 114 tested positive for HPV and 72 tested negative for HPV. Of the HPV positive cases, specific HPV types were identifiable in 109 while in the remaining five, no specific HPV could be identified (HPVX). HPV specific types identified were16, 18, 31, 35, 39, 44, 45, 51, 52 and 70. These occurred as single infections in 105 cases, double infections in three cases and triple infections in one case. HPV 16 was the most prevalent in SCC (47.8%), while in ADC HPV 18 was the most prevalent (50%).

A high proportion of cervical carcinoma tested from archival FFPE specimens harboured HPV, with more than 70% of the positive cases being HPV 16 or 18.

Our prevalence rate of 59% is somewhat lower, but is higher than that found in cervical cancer cases from Mauritius [107]. However, our overall prevalence of the two major HPV types (16 and 18) was 75%, which is similar to the recent world study by Castellsague et al. [22] and from Algeria [108] and the results from Scotland [109]. Similar low detection rates of HPV in archival cervical tissue have been reported recently from Norway [110]. Further, our negativity rates could also attributed to a very high percentage of necrosis in some of the paraffin embedded tissue and also among samples that were collected before the 90s. Formalin fixation has been shown to affect DNA [111].

The results of the study by Castellsague et al. [22], which pooled data of eight case control studies conducted in North Africa (Algeria, Morocco), South America (Brazil, Paraguay, Peru) and South East Asia (India, Thailand, Philippines), showed HPV 16 prevalence of 52.1%, HPV 18 at 39% and HPV 45 6.2%. However, regional differences were noticeable with a high prevalence of HPV 16 in North Africa and South America, while in South East Asia, there was a high prevalence of HPV 18. In this study, the prevalence of HPV 16 is rather low, while the HPV 18 prevalence is much higher than those from North Africa and South America.

In the study of Bosch et al. [17], HPV 16 occurred in 58.8% and 52% of squamous carcinomas and adenocarcinomas respectively, while the prevalence of HPV 18 was 39% in adenocarcinomas and 18% in squamous carcinomas. Our data shows a lower prevalence of HPV 16 in both squamous and adenocarcinomas, but a higher prevalence of HPV 18 in both tumours. We also did not observe types 31, 39 and 52 in adenocarcinomas, suggesting that these types may not be associated with a risk of adenocarcinomas, which is in accordance with previously reported data [17, 26]. The absence of these types was also found by other investigators [27, 108].

7.2 PAPER II

Twenty six (26) cases of cervical adenocarcinoma, which had been tested for the presence of HPV DNA were subjected to immunohistochemistry with p16, CEA, vimentin, ER and PR. 13 of these were negative for HPV. Over-expression of p16 was observed in 15 of 26 ADC. Of the cases that were p16 positive, eight were HPV positive, while the remaining seven were HPV negative. CEA was positive in 12 of 25 cases. Among the CEA positive cases, seven were HPV positive, and five were HPV negative. All five CEA positive and HPV negative cases had histological features of endocervical ADC, suggesting an endocervical origin. Vimentin

expression was positive in 6 out of 26 ADC, three of which were HPV positive and the other three HPV negative. Of the three HPV negative and vimentin positive ADC, one had a mucinous endocervical pattern, and the other two were of nonspecific features, most likely of endometrial origin. ER was not detected in any of the samples, while PR was positive in two cases, both HPV positive. These two are most likely of endocervical origin. The other five cases which were negative for all the markers, two had features of endocervical origin, and two others had features suggestive of endometrial origin, while the remaining one had nonspecific features. The expression of the different antibodies did not differ between the HPV positive and HPV negative cases.

The results showed that use of immunohistochemisrty in differentiating HPV negative cervical adenocarcinoma appears nonspecific, but some may be of value.

Compared with RIS TT study, which used similar HPV testing methods [112], the HPV positive rate in these series of ADC is low. The low detection rate could be due to denatured DNA by formalin fixation [111]. With regard to p16, only 57% of our cases were positive, suggesting some of the cases may not be of cervical origin. Some of these p16 negative ADC could be of endometrial origin. However, p16 cannot be considered as an ideal marker for HPV, although some authors suggest that it is more sensitive than ISH [113]. Moreover, cases of p16 positivity has been reported in some HPV negative ADC, suggesting HPV independent mechanisms of p16 over-expression as reported for Bowen's disease [45, 114]. Some endometrial carcinomas have also been found to express p16. Among the 13 HPV negative ADC, expression of CEA indicated they are more of cervical origin. Besides, CEA has been suggested as a marker of reserve cells [115]. Cervical carcinoma arising from reserve cells are usually HPV positive, while those from cervical epithelium are HPV negative [115]. The HPV status could be explained by the fact that there are two types of cervical ADC, one arising from reserve cells, and the other arising from endocervical epithelium. This is supported by the findings of Smedts

et al. [116], who found two phenotypically distinct types of AIS, that is AIS with a reserve cell marker phenotype and AIS with an endocervical glandular phenotype.

7.3 PAPER III

The study included 316 cases of invasive cervical carcinoma and 314 controls. HPV tests were done in 255 cases and 309 controls, while HIV testing was done in 293 cases and 308 controls. Of the 255 cases and 309 controls, HPV genotypes were detected in 222 cases (87.1%) and 95 controls (30.1%). HIV tests were positive in 55 cases (18.8%) and 54 controls (17.5%).

For SCC, the relative risk was statistically significantly increased for any HPV positive, high risk HPV types, HPV 18 related types, HPV 16 related types and some specific high risk HPV types as assessed by age-adjusted OR. The OR for any HPV positive (50.8, 95% CI: 25.8-113.9), single infections (33.3, 95% CI: 20.2-56.7), HPV 16-related types (11.6, 95% CI: 7.5-18.5), HPV 18-related types (9.4, 95% CI: 5.3-17.4), and high-risk types (64.5, 95% CI: 35.4-126.6), in particular HPV 16 (29.1, 95% CI: 15.4-60.7), HPV 18 (9.6, 95% CI: 4.6-22.3) and HPV 45 (58.7, 95% CI: lower limit >7.9).

The OR for ADC were statistically significantly increased for any HPV positive (12.9, 95% CI: 5.0-41.8), single HPV infections (13.7, 95% CI: 6.0-34.0), HPV 16-related (3.6, 95% CI: 1.5-8.2), HPV 18-related infections (22.8, 95% CI: 9.3-58.9), high-risk HPV infections, in particular HPV 16 (11.6, 95% CI: 4.3-31.8), HPV 18 (18.5, 95% CI: 6.6-54.4) and HPV 45 (297, 95% CI: lower limit >26.4).

Multiple infections and infections with low-risk HPV were not associated with increased ORs for both SSC and ADC. Adjustment for HIV as well as CD4 count decline did not change the OR estimates substantially with the different HPV types.

Overall, there was no effect on the relative risk of SCC with regards to most HPV types. HIV associated ORs for SCC were statistically significantly increased for low-risk HPV and multiple HPV infections.

Analysis of HPV relative risk in subgroups of HIV negative and HIV positive showed as having a statistically significantly increased OR for most HPV types, with the exception of HPV 18-types, which were only associated with an increased risk in HIV negative.

The results showed high relative risk of both squamous cell carcinoma and adenocarcinoma of the cervix with HPV 16 and 18, and moderate relative risk with HPV 45. Concurrent infection with HIV did not increase the relative risk with these three HPV types, but was associated with statistically borderline increased relative risk of squamous cervical carcinoma only with low-risk types and multiple infections with HPV types.

The HPV types associated with both SCC and adenocarcinoma risk in our study has also been found to be associated with the most invasive cervical carcinoma in other regions of the World [26, 117]. The relative risks also varied in the two histological types, namely SCC and adenocarcinomas. As in some previous studies, we also found HPV 16 predominating in SCC and HPV 18 in adenocarcinomas [118], although de Cremoux found that up to one third of cervical carcinomas were not associated with HPV 16 or 18 [119].

The high OR for SCC associated with HPV 16 in our study is similar to that reported by Chen et al. [120]. In addition to HPV 16, they also found an increased OR for SCC associated with HPV 52 (OR 3.04, 95% CI: 1.42-6.47) and HPV 58 (OR 5.22, 95% CI: 2.07-13.19), which was not the case in our study. Another exceptional finding was from Mexico, in which the OR for HPV 18 was higher than that of HPV 16 [23], although a similarly high OR for SCC associated with HPV 18 had been reported previously [121-123]. Some authors have found low-risk HPV in

SCC [21, 124]. For adenocarcinomas, the OR for HPV 18 in our study was lower than that reported by in a pooled case-control study by Castellsagué et al. [22].

The different relative risks of cervical carcinoma associated with HPV in many studies could be due to differences in HPV prevalence [17], which may affect risk [125]. Another possible explanation is that there are differences in the pathogenesis of the two major types of cervical carcinoma [126]. Studies by Smith et al and Madeleine et al found increased risk of SCC associated with trichomonas, but no increased risk of adenocarcinoma. Another possible factor is smoking [127], although this is unlikely in Uganda, where the prevalence of smoking is very low [128, 129]. The low relative risk of cervical carcinoma with other high-risk HPV types is supported by the low prevalence of these types in cervical carcinoma from various studies [17, 130].

Although many studies have shown increased risk of cervical carcinoma as a result of HIV infection [87, 131, 132], some have found low relative risk [90, 133]. Of interest was a lack of association between HIV and cervical cancer in general. After adjusting for HPV infections, the association between HIV and SCC was not statistically significant with most HPV types, except low-risk HPV and multiple HPV infections. For adenocarcinoma, there was no association.

Our study did not corroborate the hypothesis that HIV infection increases a woman's risk for invasive cervical carcinoma. The OR associated with different HPV infections were similar in both HIV positive and negative women, except for low-risk HPV and multiple infections for SCC. This could be attributed to impaired immunity as previously suggested [134, 135].

7.4 PAPER IV

Overall, more than 80% of both fresh and formalin fixed tissues were positive for HPV, giving and overall agreement in HPV detection in both specimens at 86%, which is statistically significant. In both types of specimens, single infections were predominant, with HPV 16

accounting for 47% in both specimens and almost identical proportions of HPV 33, 52 and 58, while the proportions of the other HPV types were different. When analyzed for overall agreement, complete agreement was found to be 59.7%, partial agreement 5.3% and complete disagreement 35.0%. Comparing HPV detection by histological type of cervical carcinoma, adenocarcinomas were more likely to be HPV negative than squamous cell carcinoma in both fresh and fixed tissue.

The results of our study showed comparable HPV detection in both fresh and FFPE tissue by SPF10 LiPA method. Some previous studies that compared the paraffin embedded tissues with exfoliated cells or cervical scrapes and not frozen specimens found high agreement [136-138]. However, these were cases of pre-neoplastic lesions, not invasive cervical carcinoma. A comparison of cervical scrapes and FFPE tissues from invasive cervical carcinoma cases found very high HPV type specific concordance [139]. The SPF10 which we used is more sensitive, but less specific than the GP5 and GP6 method [140]. Other previous studies also found low HPV DNA prevalence in ADC of the cervix [25, 141, 142], the low detection being attributed to low viral load, fewer episomal copies or loss of viral genome during integration in cervical adenocarcinoma as suggested by Park [143].

8 GENERAL DISSCUSSION

The findings of this thesis have highlighted various aspects of cervical carcinoma in Uganda. One of the areas is HPV types associated with cervical carcinoma in Uganda. The results show that the current vaccine maybe able to prevent a high proportion of cervical carcinoma in Uganda. This is consistent with findings of studies from other previous investigators [17, 22, 108]. Besides, our results show that HPV types associated with cervical carcinoma appear to be stable over the years. This suggests that the current available vaccines could probably be of use for many years ahead, substantially diminishing the cervical cancer impact in the country.

Another aspect was on the risk of cervical carcinoma associated with HPV. The relative risks were particularly high with HPV 16, 18 and 45. This is similar to results of some previous studies [26, 117], but also differs from those reported from some areas like Mexico [23] and Taiwan [120]. The implication of results from this thesis suggest that in Uganda, women who are positive for HPV 16,18 or 45 may need more frequent screening compared to those with other high-risk HPV. The results of this thesis also highlight the fact that there are variations in relative risks of cervical carcinoma, suggesting possible oncogenic differences of HPV isolates from different areas or differences in regional cofactors of cervical carcinoma.

One of the factors that have affected cervical carcinoma risk has been the AIDS epidemic. Several studies have shown an increased relative risk of cervical carcinoma as a consequence of HIV infection [87, 131, 132], although others indicated no increased risk [90, 133]. However, our study did not corroborate the hypothesis that HIV infection would increase a woman's risk for invasive cervical cancer, and the OR associated with different HPV infections and cervical cancer risk were similar in HIV positive and negative women, except for low-risk and multiple infections for SCC. Our findings have shown that in the setting of HIV, SCC and not

adenocarcinoma is likely to arise as a consequence of some HPV types. Other studies need to be done to elaborate on these aspects.

This has implications with regard to cervical carcinoma screening in HIV positive women, especially those born with HIV. In addition, it suggests that ADC may not be influenced by HIV infection. Although other previous studies have indicated increased relative risk of cervical carcinoma as a result of HIV infection in Africa [87, 132], this could be due to the fact that there was no adjustment for HPV.

Further insight was also demonstrated in the use of different samples for HPV testing. While other investigators compared fresh tissue with cervical scrapes, we were able to compare fresh samples and paraffin embedded tissue from same patient. This indicates that both specimens gave comparable results, and suggests that FFPE tissue could be used, especially in countries with low income.

Comparable detection rates of HPV in FFPE samples and fresh samples demonstrated in this thesis indicate that the SPF 10 method is robust. Though other investigators using FFPE tissues found low rates of HPV detection [107, 110], this may be attributable to denatured tissue. It is known that DNA extracted from FFPE tissues are usually at low concentration and fragmented [111, 144]. The low detection rate of HPV in adenocarcinoma from both specimens is of interest and deserves some comments. One plausible explanation is that adenocarcinomas are a heterogenous group of tumours, some not being related to HPV. This is supported by results of Smedts et al. [116], who showed that there are two phenotypic types of AIS. The findings also suggest that adenocarcinomas and SCC have different pathways of HPV associated carcinogenesis.

In this thesis, we were also able to demonstrate that the use of different antibodies maybe useful in distinguishing cervical adenocarcinomas from endometrial adenocarcinomas. This is of

particular interest, since an increase of cervical adenocarcinomas have been reported by some authors of late [145, 146]. Different antibodies could be used to find out if the increase is of cervical or of endometrial types. Besides, it is useful to find out if these cases are related to HPV by used of HPV DNA and p16, as suggested by Yemelyanova et al. [147]. Use of HPV DNA detection could also be extended to epidemiological studies of cervical adenocarcinomas to minimize misclassification. Our study is unique because many cervical cancer samples were negative for HPV, considering that HPV is a necessary cause of cervical cancer. This is probably because of problems during the original fixation of tissue in Uganda. Our working hypothesis was that immunohistochemical markers could be useful in distinguishing the tissue of origin i.e cervical or endometrial of samples initially diagnosed as adenocarcinomas (ADC) of the cervix that were HPV negative. The fact that the samples used were from Uganda is also of interest, since there are very few pathological studies on cervical carcinoma emanating from this country, where the prevalence of diseases is amongst the highest in the world [3]. Problems in classification of tumours found in the Ugandan setting are probably representative of problems found in several other sub-Saharan countries.

8.1 METHODOLOGICAL ISSUES

8.1.1 Study subjects

The aim of the thesis was to provide more information of different aspects of cervical carcinoma in Kampala, Uganda. The study assessed the types of HPV in cervical carcinoma and the use of different antibodies to ascertain the nature of HPV negative cervical carcinoma, compared the detection of HPV in fresh and formalin fixed paraffin embedded tissue and finally estimated the risk of cervical carcinoma associated with HPV and HIV infection. Compared with previous studies, our study had a large sample and was carried out in an area with a high incidence of cervical cancer [2].

The selection of study subjects was based on selected criteria. All the histological materials were verified as invasive cervical cancer. Women selected as cases were all histologically confirmed as invasive cancer, and control women were those without cervical abnormalities cytologically. In addition, the women were interviewed using a standard questionnaire. All laboratory samples were obtained and processed using standard operating procedures.

8.1.2 Internal validity

To assess internal validity, we evaluated possible bias, confounding and chance.

8.1.2.1 Bias

8.1.2.1.1 Selection bias

To avoid this, we did not set any criteria for selection of samples, with all potential participants having an equal chance. Since this was a case-control study done at a Ministry of Health facility, cases of cervical carcinoma who opted to use private or non-governmental health facilities could lead to some selection bias.

8.1.2.1.2 Misclassification bias

For the case control study, we had strict guidelines on classification of exposure variables. Laboratory samples were processed under standard conditions with clear definition of positive or negative outcomes. The technical staff who did the laboratory analysis were blinded. Despite these measures, some misclassification could have occurred. Due to limited resources, classification of cervical carcinoma was based only on routine haematoxylin and eosin (H&E) stain, which could have led to some misclassification of some cases, especially adenocarcinoma. In order to avoid this, HPV DNA and immunohistochemistry using p16 and hormonal receptors have been applied with good results [147]. For control women, cervical smears were obtained and examined to ascertain the cervical condition. In addition, some cases of HPV might not have been detected, since the methods used utilizes only known HPV primers [10]. However, this may not affect the results significantly.

Use of HIV rapid tests might not have identified some infections. However, this would affect both cases and controls, and therefore may not affect the results adversely. Another limitation of the study was that we could not differentiate HIV into types 1 or 2, as has been done in studies from Senegal [24, 86, 132].

For diagnosis of cervical carcinoma, all were based only on routine H&E. These could have led to some occult mucus secreting adenocarcinoma being misclassified as SCC [148]. Besides, some endometrial carcinoma could have been misclassified as endocervical carcinoma since immunohistochemistry was not done on most cases of adenocarcinomas.

Measurement of immunity is complex process. Depending on available resources, a number of measurements can be done. With limited resources, one can use CD4 as a measure of immunity. Although it is not precise, it has been used within a number of studies.

8.1.2.2 Confounding

Like most epidemiological studies, confounding has to be minimized in Article III. To minimize this, we adjusted for the effects of possible confounders in the analysis in the case control study. One of the confounders was age. We therefore fitted age-adjusted models for each HPV type or groups of HPV types, as well as models also adjusting for HIV (positive or negative) and CD4 count per 100 cells, as a linear continuous variable(s). Adjustment for HIV did not affect the OR with many HPV types. In addition to adjusting, we also analyzed the data in subgroups of HIV negative and HIV positive and found no significant differences in the OR of most HPV types. Immune status as assessed by CD4 counts as a possible confounder was also adjusted for with no significant effect on the OR. The fact that adjusting for HIV and its immune effects on the relative risk of cervical carcinoma with different HPV implies a central role of HPV in cervical carcinogenesis.

Confounders associated with sexual lifestyles are more of a proxy marker for the acquisition of HPV. Studies have shown that two out of three people who have sexual intercourse with a HPV

positive person acquire the infection [72].We therefore thought it was not important to adjust for these factors.

Some possible confounding could not be controlled, as the information was not available in our questionnaire. One example, is exposure to indoor air pollution caused by smoke from cooking fire, which has been associated with cervical carcinoma [149]. Like in most developing countries, the use of firewood for cooking is highly common in Uganda. However, this would have affected both cases and controls. Another factor is education level. However, a pooled analysis showed that there were no differences in HPV positivity by education level, and concluded that the excess of cervical cancer found in women with little education is not due to an excess of HPV prevalence, but rather by early events which may modify the cancer causing ability of HPV [150].

8.1.2.3 Chance

To assess whether our results could be due to chance, we used statistical tests to evaluate this. We used 95% confident intervals and p-values. Results were the 95% CI included 1 or p-value >0.05 were considered to have occurred by chance.

8.1.3 External validity

Results in this thesis were obtained from analysis of archival materials as well as samples got prospectively. The samples were obtained in a hospital setting. Although the hospitals serve the population from whom the samples were obtained, one has to be cautious in extrapolating the findings to the overall population. However, we think the results give some baseline information and could be applicable to the population, since the prevalence of HPV in controls is similar to the one of women from East Africa [151].

8.1.4 Limitations of laboratory methods

The SPF10 LiPA system we used has a limited HPV detection, because its use is based on probes of known HPV types in detection methods [10].

Another limitation of the study was that we could not differentiate HIV into types 1 or 2, as has been done in studies from Senegal [24, 86, 132]. In addition, some antigens could have been denatured as a consequence of uncontrolled fixation of tissues.

8.2 INTERPRETATION AND IMPLICATIONS OF FINDINGS

8.2.1 Patient care

From results of Paper 2, the use of some selected antibodies may be useful in differential diagnosis of cervical adenocarcinoma, especially cases with unusual morphological features. The use of FFPE tissue could also be used for detecting HPV in clinical settings. For example, if a woman has been diagnosed with preneoplastic lesions and a biopsy has been obtained, the FFPE could be used for HPV testing.

With the availability of information on HPV, a number of women may wish to know their HPV status. Since our results show that HPV 16, 18 and 45 are associated with an unduly high-risk of cervical carcinoma, women who are found positive for these HPV may need more frequent screening.

HPV testing could also be done in cases of head and neck carcinomas. A number of studies have shown that HPV positive head and neck carcinomas have better prognosis than those HPV negative carcinomas [152-154]. HPV testing on FFPE samples from patients with carcinoma of head and neck could therefore be performed to provide information on prognosis.

8.2.2 Public health policy

Article I showed that HPV 16 and 18 accounted for 84% of all HPV positive cervical carcinoma. The use of the available HPV currently on trial could probably prevent most cases of cervical cancer in Uganda. Screening for cervical carcinoma could be supplemented with HPV testing if resources are available.

Our results from article II show that use of antibodies may be useful in sub-classification of adenocarcinomas of cervical origin. Depending on available resources, some guidelines need to be developed so that women diagnosed with adenocarcinomas of the cervix may require additional investigation to exclude endometrial origin. In addition, the use of HPV tests as tools for screening for cervical carcinoma has to be used cautiously, since the risk of cervical adenocarcinoma would still be there.

Results from Article III showed no excess risk of cervical carcinoma due to HPV 16 and 18 in HIV positive or negative women. In view of these results, both groups of women should have similar PAP checks if they are positive for HPV 16 or 18. However, HIV positive with low-risk HPV and multiple HPV infections may require more frequent PAP checks, since the relative risk of cervical carcinoma is marginally elevated in these groups.

With the introduction and availability of HAART in Uganda, there are now a number of adolescents who are HIV positive. These HIV positive virgins may need special attention with regard to HPV. Questions which need to be addressed include whether they should be vaccinated with the available vaccine, and when, and how should cervical cancer screening in these groups be done.

Results of article IV showing comparative similarity between formalin fixed paraffin embedded specimens and fresh samples also have some implications. Guidelines need to be developed on fixation of tissues. This is especially important since the tissues could also be used for other specialized laboratory tests.

8.2.3 Research

Results from Article I showed more than 50% of cervical carcinomas from Uganda were due to HPV 16 or 18, with the remaining due to other HPV types. Although this indicates that the current vaccine could prevent most of these cases, the occurrence of cervical carcinomas due to other HPV types raises some concern. HPV types distribution has been shown to vary in different areas of the world [17]. In Uganda, it would be of interest to study the HPV prevalence in different areas. Studies from China by Chen et al. [25] showed that women with SCC aged 34 years or less had a higher proportion of HPV 16 compared to those aged over 50 years in whom HPV 52, 58 and 39 were more common. The results of the study from China tend to suggest that some HPV types may be acquired later in life. Studies would need to be done in Uganda to ascertain if the prevalence of HPV types in various age groups also differs. In addition, the nature of uncharacterized HPV types (HPV X) requires more exploration.

Results of Article IV showed fairly comparable results from the use of fresh and FFPE for HPV detection. The available FFPE tissue could be used to study a number of diseases related to HPV. A number of samples have been used for detecting HPV [137, 138]. Compared with tissue samples, exfoliated cervical cells or scrapes may be contaminated with vaginal samples and hence unreliable. Our study therefore adds valuable information to the small number of studies on the concordance of different genotyping methods for HPV detection in biological specimens stored under different conditions. Besides, our results suggest that more refined methods for HPV detection needs to be developed for cervical adenocarcinomas. Another area of research also requiring attention is whether HPV integration differs between cervical squamous cell carcinomas and adenocarcinomas. Lastly, research on HPV related diseases could be done using FFPE specimens.

Results from Article II reaffirm that p16 could also be used as a surrogate marker for HPV in research of HPV related tumours. Redman et al suggested that p16 could be used to distinguish

between HPV related lesions and non HPV related lesions [155]. This could also be extended to epidemiological studies of cervical adenocarcinoma, where the use of a panel of antibodies would be advisable to avoid misclassification.

In view of the increased relative risk of cervical squamous cell carcinoma associated with low-risk HPV and multiple HPV infections associated HIV infection, more research needs to be done. It would be worthwhile to investigate if the risk predominantly is confined to only squamous cell carcinoma. Further, there is also a need to assess if there are some particular risk factors associated with this increase. Lastly, the lack of increased risk of cervical adenocarcinoma as a consequence of HIV infection needs to be investigated.

8.2.4 HPV reference laboratory to be set in Uganda or in the East African Region

Investigations of HPV associated diseases would probably differ from one country to another based on health priorities. In view of these, each country or regional countries could set a central laboratory unit for HPV. This central laboratory could then liaise with other well-developed HPV laboratories for quality control and referral of some cases.

8.2.5 HPV vaccination

From results of this thesis on HPV prevalence in cervical carcinoma specimens, one could postulate that the current HPV vaccines may be able to eradicate about 70% of cervical cancer in vaccinated females. However, a sizable percentage of vaccinated women may still remain at risk of cervical carcinoma from the other HPV types, since cervical carcinoma have occurred in women who were vaccinated [156].

9 CONCLUSIONS

9.1 AIM 1: To ascertain the types of HPV distribution in invasive cervical cancer in Uganda.

Conclusions: Our results confirm the role of HPV 16 and 18 in about 75% of cervical cancer in the Ugandan population and suggest that the currently available HPV vaccines against HPV 16 and 18 could possibly prevent the majority of invasive cervical cancers in Uganda.

9.2 AIM 2: To ascertain the usefulness of immunohistochemistry profiles in tissue microarrays in archived samples of adenocarcinoma of the uterine cervix that had been tested for HPV DNA, and in particular to assess if it would be possible to define the tissue of origin of HPV negative adenocarcinoma sample.

Conclusions: There is overlap in expression of p16, CEA and vimentin between HPV positive and HPV negative cervical ADC.

Some HPV negative ADC diagnosed as cervical ADC may be of endometrial origin.

CEA and vimentin may be of value in distinguishing HPV negative cervical tissue from endometrial ADC.

9.3 AIM 3: To estimate the relative risk of cervical carcinoma in association with different HPV types and HIV in Kampala, Uganda.

Conclusions: The OR for squamous cell carcinoma and adenocarcinoma of the cervix in Uganda were increased in women infected with HPV, in particular single HPV infections, infections with HPV16- and 18-related types, infections with high-risk HPV types, specifically with HPV16, 18 and 45.

Adjustment for HIV infection as well as CD4 decline did not change the OR estimates substantially with the different HPV types.

Cervical cancer was not statistically associated with HIV infection per se, and HIV positive women did not have a higher OR for cervical cancer in relation to most HPV types, except for low-risk and multiple infections in SCC.

9.4 AIM 4: To compare HPV detection in fresh and paraffin embedded cervical carcinoma specimens.

Conclusions: No differences in HPV positivity were observed between fresh tissue and paraffin embedded blocks globally.

For specific types, only 59.7% of the cases had complete agreement on the HPV detection.

Although HPV DNA detection was lower in ADC as compared to SCC, these differences were not statistically significant, $p > 0.05$.

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Human Papillomavirus type distribution in invasive cervical cancer in Uganda

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Abstract

Background: We conducted a study aiming to describe Human Papillomavirus (HPV) type distribution in invasive cervical carcinoma in Uganda.

Methods: 191 archival cervical carcinoma samples diagnosed in the Department of Pathology, Makerere University in Kampala between 1968 and 1992 were analysed using a sensitive PCR-Reverse Hybridization Line Probe Assay.

Results: Out of the 186 cases of confirmed invasive cervical cancer in the study paraffin blocks, 114 were positive for HPV DNA. Specific HPV genotypes were identifiable in 109 cases: HPV 16, 18, 31, 35, 39, 44, 45, 51, 52 and 70. These occurred as single infections in 105 cases (96.3%) and as multiple infections in 4 cases (3.7%). HPV 16 or 18 accounted for 80% (84/105) of cases with single infection.

Conclusion: The results of this study confirm the role of HPV 16 and 18 in cervical cancer pathogenesis in the Ugandan population. The results suggest that the currently available HPV vaccines against HPV 16 and 18 could possibly prevent the majority of invasive cervical cancers in Uganda.

Background

Clinical and epidemiological studies have clearly established that infections by certain human papillomaviruses (HPVs) types are causally linked to cervical cancer development [1-5]. Among the high risk HPV types, HPV 16 and 18 are recognized as the main causes of invasive cervical cancer and its precursor lesions [3]. These two viral types were found in most cases of invasive cervical cancer from 22 countries around the world [4]. HPV 16 tends to predominate in squamous cell carcinomas whereas HPV 18 often predominate in adenocarcinomas [4,6-8]. Some studies have also shown that there are geographical variations in HPV type distribution [1,4,9,10]. Finally, recent data shows that HPV immunization offers the greatest possibility for prevention of cervical cancer and has become an important health priority to many govern-

ments and health organizations worldwide. HPV vaccines that are currently available include VLPs for HPV 16 and 18 [11,12] as well as HPV 11 and 6 [13]. However, geographical variations in HPV type distribution might influence the currently available HPV vaccine efficacy. Compared to other continents (such as South America and Asia), there is relatively little information about HPV types in invasive cervical cancer in Africa. We conducted a study aiming to characterize HPV type spectrum in the archival specimens from cervical cancer cases from Uganda population including cases diagnosed over 34 years.

Methods

191 samples from patients with histological diagnosis of invasive cervical carcinoma from 1968 to 1992 were obtained from the archives of the Department of Pathology, Makerere University in Kampala, Uganda. These samples accounted for less than 5% of cervical carcinomas diagnosed during the study period. Nine cases (4.7%) were from the period of 1968 to 1969, 66 (34.6%) cases from 1970–1979, 74 (38.7%) from 1980–1989 and 42 (22.0%) were from the period 1990–1992. Until the early nineties, the Department of Pathology received most specimens for histopathology services from all hospitals and health facilities in the country.

Samples were received in formalin, which may not always have been buffered. Tissue specimens were usually processed within 24 hours of their arrival to the hospital, but for a fraction of samples transported from remote parts of the country, longer times may have been required. Samples were selected randomly from the register of Pathology Department starting in 1968; we oversampled of adenocarcinomas (1:1) because of the special interest to compare genotype distribution in the two major histologies of cervical cancer.

When at the testing laboratory, the paraffin embedded tissue blocks were re-embedded in fresh paraffin wax and four sections (sandwich method) were cut for testing under strict conditions to avoid potential contamination. The first and the last sections were stained with Haematoxylin and Eosin (H&E) to confirm diagnosis and to ascertain the suitability of the tissue to continue for HPV testing. The sections in-between were collected in a screw-top Eppendorf tube for HPV testing. A tissue-free paraffin block was cut after each study block to avoid any HPV carry-over from block to block. A new blade was used for each block and the microtome was cleaned with a vacuum cleaner, Histoclear II and 70% alcohol. To further control for possible sources of contamination, paraffin blocks containing non HPV related lesions (appendicitis, lung tumour, ovary, lymph node etc.) were included blindly in the process at a ratio of 5%, and blank paraffin sections were simultaneously tested.

Proteinase K (Sigma) digestion for 16 hours at 56°C temperature was used to obtain a tissue lysate containing DNA from the paraffin inner 5 μm sections. SPF10 PCR was performed using 10 μl of a 1:10 dilution with water of the tissue digest in a final reaction volume of 50 μl [14]. The amplified PCR products were tested using a probe hybridization with a cocktail of conservative probes recognizing at least 54 mucosal HPV genotypes in a microtitre plate format for the detection of HPV DNA. Optical densities (OD_{450}) were read on a microtitre plate reader. HPV DNA positive samples were subsequently analysed by HPV SPF10-$LIPA_{25}$ (version 1: produced at Labo Biomedical Products, Rijswijk, The Netherlands) [14], a reverse hybridization technique that detects 25 high-risk and low-risk HPV types (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, 74). The sequence variation within the SPF10 primers allows the recognition of these different HPV genotypes, except for the types 68 and 73 as their interprimer regions are identical and cannot be distinguished on this test. After PCR, 10 μl of the amplimers was used to perform reverse hybridization for HPV genotype identification. The positive hybridization on the strips is visualized as a purple band by means of a precipitating colour substrate on the probe site. Specimens that were HPV/DNA positive but did not hybridize with any of the 25 probes were coded as HPV type X (unknown type). Amplification of a fragment of the human β-globin gene was performed in all HPV/DNA negative samples to assess DNA quality. HPV type-specific distributions were calculated among HPV positive women. All SPF10-LIPA25 PCR detection and typing was performed at the facilities of DDL Diagnostic Laboratories (DDL, Voorburg, Netherlands) and at Institut Català d'Oncologia (ICO, Barcelona). HPV detection was evaluated by histology classification (adenocarcinoma vs. squamous cell carcinoma), year of diagnosis and presence of necrosis in the tissue.

Ethical approval for the reported study was obtained from Ethical Committee at Makerere University, Kampala, Uganda.

Results

Of the 191 paraffin blocks corresponding each one to a cervical cancer cases, 186 harboured invasive cervical carcinoma cells and, according to protocol, were considered suitable for HPV detection. Five were excluded due to extended necroses seen on the histological slide. 146 were diagnosed as squamous carcinomas, 35 were adenocarcinomas, 3 adenosquamous and two were undifferentiated carcinomas. Out of these, 114 were HPV positive and in 72 HPV could not be detected. All the negative samples

Table 1: HPV/DNA detection by cervical cancer histological types

	HPV negative[a]	HPV positive[b]	Total
Squamous carcinoma	54	92	146
Adenocarcinoma	15	20	35
Adenosquamous	1	2	3
Undifferentiated carcinoma	2	0	2
Total	72	114	186

[a] HPV not detected. All these samples were human β-globin gene negative, and therefore inadequate for HPV determination
[b] All β-globin gene positive

tested negative for the presence of β-globin gene indicating a poor quality tissue. Table 1 shows the overall β-globin gene and HPV positivity results according to histological types. There was no statistical differences between histological type and overall HPV infection (Chi2 = 3.64, p = 0.3). There was stability of genotype distribution of HPV 16 and HPV 18 over time, taking into account histology.

Specific HPV types were identifiable in 109 cases, while in the remaining five cases no specific HPV types could be identified (Type X). Ten specific HPV types: 16, 18, 31, 35, 39, 44, 45, 51, 52 and 70 were identified. These occurred as single infections in 105 cases, double infections in three cases and triple infections in one case. Analysis of the HPV genotypes distribution among the HPV positive cases showed that HPV 16 and 18 were the most frequent types, followed in descending order by HPV 45, 31, 35, 51, 39 and 52. Table 2 shows the distribution of HPV infections and histological types.

In single infections, type 16 occurred in 51 of the cases, type 18 in 33 cases, type 45 in 11 cases, type 31 in four cases, type 35 and 51 in two cases, types 39 and 52 each in one case. The three double infections were found to be combinations of types 16/45, 16/51 and 45/70 while the triple infections included the HPV types16/44/52. When the analysis was restricted to the histological types of cervical carcinoma, HPV 16 was the most frequent in squamous cell carcinomas (47.8% of cases) while in adenocarcinomas, HPV 18 was the most prevalent type (50%). Of the squamous cell carcinomas, 44 of the cases

Table 2: HPV type distribution according to histological types

HPV types	Squamous carcinomas	Adenocarcinomas	Total
	Infection with single HPV		110 (96,5%)
	N (%)	N (%)	N (%)
HPV 16	44 (47.8)	7 (35.0)	51 (44.7)
HPV 18	21 (22.8)	10 (50.0)	33 (28.8)[a]
HPV 16 or 18	65 (70.6)	17 (85.0)	84 (73.7)[a]
HPV 31	4 (4.3)	0	4 (3.5)
HPV 35	2 (2.2)	0	2 (1.8)
HPV 39	1 (1.1)	0	1 (0.9)
HPV 45	10 (10.9)	1 (5.0)	11 (9.6)
HPV 51	1 (1.1)	1 (5.0)	2 (1.8)
HPV 52	1 (1.1)	0	1 (0.9)
Type X	4 (4.3)	1 (5)	5 (4.4)
	Infection with multiple HPV		4 (3.5%)
HPV 16/45	1 (1.1)	0	1 (0.9)
HPV 16/51	1 (1.1)	0	1 (0.9)
HPV 45/70	1 (1.1)	0	1 (0.9)
HPV 16/44/52	1 (1.1)	0	1 (0.9)
Total	92	20	114[a]

[a] Includes two additional cases of adenosquamous carcinomas.

presented with HPV type 16, 21 of the cases with type 18, 10 with type 45, four with type 31, two with type 35, three single infections with types 39, 51, and 52, three double infections and one triple infection. In the adenocarcinomas, 10 were found to have HPV type 18, 7 type 16, one type 45, and one type 51. Concurrent CIN lesions were seen in 18 cases with single HPV infections and one case with multiple HPV types. No differences in HPV detection were observed between the cases with and without preneoplastic lesions (Table 3).

Discussion

This study shows that HPV/DNA was detected in 114 out of 186 invasive cervical cancer samples, giving an overall HPV prevalence of 61.3% of the samples. If the β-globin gene results are taken into account, then HPV was detected in 100% of the samples from which cellular DNA was obtained.

Specific HPV genotypes were identified in 109 cases using the highly sensitive SPF10 LiPA technology. Most of the infections were due to single HPV types with only four cases due to multiple infections. HPV 16 and 18 accounted for more than 70% of the cases, while the remaining were due to the other HPV types, with HPV 45 constituting a major proportion. In squamous cell carcinomas, single infections due to HPV 16 and 18 occurred in 70% of cases, while in adenocarcinomas these two types occurred in 85% of cases. Of particular note is the high proportion of squamous cell carcinomas (10.9%) due to HPV 45. Almost all infections were high risk HPV types, with exception of two low risk HPV (44 and 70) which were seen as part of multiple infections. There were only 5 samples classified as HPV X (HPV/DNA positive and genotype negative); we hope to be able to genotype these samples in the future. HPV 16 and 18 were the 2 most common types identified throughout the study period indicating viral genotype stability in an era in which HIV started to emerge.

The prevalence of HPV DNA in cervical cancer varies in many studies, with some investigators reporting almost 100% [2,4]. This is probably attributable to the efficient extraction of intact DNA from the fresh cervical scrapes or cervical tissues used in these studies. The prevalence of HPV positive samples in our study is relatively low, but higher than that found in cervical cancer cases from Mauritius [15]. Similar low detection rates of HPV in archival cervical tissue have been reported recently from Norway [16].

Table 3: Multiple HPV genotypes in invasive cervical carcinoma cases with and without concurrent CIN lesions

	HPV + (1 type)	HPV + (>1 type)	Total
No CIN	87 (96.7%)	3 (3.3%)	90
With CIN 1/2/3	18 (94.7%)	1 (5.3%)	19
Total	105	4	109

$p > 0.05$

In our study, all the HPV/DNA negative samples were tested for β-globin gene. All were found to be β-globin gene negative, although the β-globin gene primer used was slightly longer than the very short primer used in the DEIA reaction. Thus, the low detection rate is very likely due to poor quality of the DNA in some archival tissue samples. It has been demonstrated that suboptimal processing of specimens in the histological process such as the use of unbuffered formalin as a fixative, which was common before 1970, may yield DNA that is unsuitable for HPV detection [16]. In a subset of 200 samples from the large international RIS HPV TT study, the HPV negative samples were further explored and 89% of them were found to be β-globin gene negative, thus attributing this relatively high rate of HPV negativity to the poor quality of these retrospective samples. It was also found that adenocarcinomas were more likely to be HPV negative than squamous cell carcinomas [17].

HPV 16 and 18 are the major aetiological risk factors for cervical carcinoma. Our overall prevalence of the two major HPV types (16 and 18) was over 70%, which is similar to the IARC multicentric case-control study by Castellsague et al. [1], the study by Hammouda et al. from Algeria [18] that both used fresh samples and GP5+/6+ PCR assay, as well as the most recently meta analysis of the published literature [9,10]. The study of Castellsague et al. [1], which provides pooled data of eight case control studies conducted in North Africa (Algeria, Morocco), South America (Brazil, Paraguay, Peru) and South East Asia (India, Thailand, Philippines), showed HPV 16 prevalence of cervical adenocarcinomas of 42.7%, HPV 18 at 31.8% and HPV45 3.8% [1]. However, regional differences were noticeable with a high prevalence of HPV 16 in North Africa and South America, while in South East Asia there was a high prevalence of HPV 18. A recent study from Mozambique [19] on invasive cervical cancer reported a similar prevalence of HPV 16 (about 47%) and of HPV 16 and 18 combined (about 71%) as in our study. Compared with the results of pooled analyses of invasive cervical cancer from different continents [9,10,20], our study presents a lower prevalence of HPV 16 (44.7%) than in Europe, America, Northern Africa and South Asia but comparable with Sub-Saharan Africa, while the HPV 18 prevalence is higher (28.8%) than in all regions [9]. Considering the adenocarcinomas only [1], the prevalence of HPV 16 in our study is lower (35.0%) than in North Africa (72.7%), South America (71.7%) and comparable with

the prevalence in South-East Asia (34.6%). The prevalence of HPV 18 in adenocarcinomas in our study (50%) is somewhat lower than in South East Asia, but more than twice higher than in the other regions.

The causes of the regional differences in HPV subtype prevalence need clarification, e.g. the results from Mauritius [15], where only HPV 18 was detected, warrants a follow up study. The proportion of HPV types in the two major histological types of cervical carcinoma, i.e. squamous cell carcinoma and adenocarcinoma have been noted to vary, although HPV 16 tend to predominate in squamous carcinomas and HPV 18 in adenocarcinomas. In the study of Bosch et al. [4] HPV 16 occurred in 51.2% and 28% of squamous carcinomas and adenocarcinomas respectively, while the prevalence of HPV 18 was 56% in adenocarcinomas and 12.1% in squamous carcinomas. In distinction from Bosch et al. results [4], our data shows a lower prevalence of HPV 16 in squamous cell carcinomas but a higher in adenocarcinomas, and a higher prevalence of HPV 18 in squamous tumours, lower in adenocarcinomas. We also did not observe types 31, 39 and 52 in adenocarcinomas, suggesting that these types may be less commonly associated with risk of adenocarcinomas which is in accordance with previously reported data [1]. The absence of these types was also found by other investigators [18,21] but not by the preliminary data of the RIS HPV TT study [22].

One of the interesting findings of our study was the five cases in which the samples were positive for HPV, but specific HPV genotypes could not be identified. This raises the possibility that there may be some unidentified HPV types or that some additional HPV types could have a wider range of probes. In a study in Mozambique [23], HPV specific types could not be identified in 19% of the HPV positive cases, and this failure was attributed to the limited number of HPV specific probes used.

Multiple infections were seen in only four HPV positive cases (3.5%) in this study. This is similar to some previous studies where the prevalence varied from 2.5% in Spain and Columbia [5], 3.9% in Thailand [6], 4.3% in Brazil [24], 7.9% in Morocco [25], and 12.9% in women with squamous tumour in Peru [26]. However, much higher rates were also reported by other workers, from 19.3% in Paraguay [27], 32.2% in Korea [28], 32% in Costa Rica [29] and 34.3% in Mozambique [30]. These differences could be due to underlying prevalence of multiple infections in the various populations or to differences in the methods used in detection and diagnosis [31].

Whether multiple HPV infections are involved in cervical cancer pathogenesis is rather unclear. Herrero et al. found that the risk of cervical cancer associated with HPV 16 alone is similar or greater than the risk associated with multiple infections (HPV 16 plus other HPV) [29]. The results of Lee et al. found multiple infections associated with increased cervical cancer risk [32]. Although the number of cases in our study is rather small, the results have some important implications. If the HPV vaccines under trial containing 16 and 18 antigens are close to 100% efficacy [11,13], then one could assume that about 70% of cervical cases would be prevented by the use of the vaccine. It also raises the question whether other HPV types should be included in future HPV vaccines. This is of concern because of multiple infections or infection with other common HPV genotypes which could account for 5% to 10% of cervical cancer cases. The results of our study are in agreement with previous reports by Hammouda et al. [18], and Castellsague et al. [1].

Our study had a number of strengths. The method we used is sensitive and has high efficacy in comparison with other methods using other general HPV primer sets [14]. In addition, extreme precautions were undertaken to avoid contamination, and stringent SOP (standard of procedures) were applied throughout the study. To avoid false positive results, controls consisting of non-tumour tissue blank paraffin sections and other negative control samples were simultaneously tested in each of the different steps of analysis.

One limitation of our study was the relatively small number of cases tested. Since the specimens came from different parts of the country, the duration of fixation were not uniform and could have contributed to some cases being negative for HPV. In addition, the use of non buffered formalin could also have deteriorated the DNA, resulting in false negative results.

In our study we did not have information on HIV serology. HIV infection is a risk factor for pre cervical cancer lesions [33,34] and also probably for invasive cervical cancer [34], although some earlier studies did not find any associations [35]. Patients with HIV seem to have infections with a broader spectrum of HPV types other than HPV 16, and more prone to simultaneous multiple HPV infections. The prevalence of HIV in Uganda could have been as high as 20% during part of the time when the samples were collected. This is based on HIV seroprevalance among pregnant women which rose from 10% in 1985 to around 30% in 1990–1992 [36]. Although this is a very high rate of HIV, we did not detect in our samples a different HPV genotype distribution of the most frequent types, neither a higher number of multiple infections.

Conclusion

The results of our study show that the HPV type distribution can be determined using existing archived biological materials, such as paraffin blocks, allowing evaluating changes over time, and reaffirm the role of HPV in cervical cancer pathogenesis in the Ugandan population. HPV 16 and 18 account for about 75% of cases while about 20% are due to other HPV genotypes, and the remaining due to multiple HPV types. The results suggest that more HPV types are yet be identified and recommend that future HPV vaccines be tailored according to local HPV type distribution.

Abbreviations

CIN: Cervical Intraepithelial Neoplasia; DDL: Delft Diagnostic Laboratories; DEIA: DNA Enzyme Immunoassay; DNA: Deoxyribonucleic acid; H&E: Haematoxilin and Eosin; HIV: Human Immunodeficiency Virus; HPV: Human papillomavirus; ICO: Institut Catala d'Oncologia; LiPA: Line Probe Assay; OD: Optical density; PCR: Polymerase Chain Reaction; RIS HPV TT: Retrospective International Study on HPV Distribution among Cases of Invasive Cervical Cancer; SOP: Standard of procedures; SPF 10-PCR: Short-Fragment PCR; VLPs: Virus-like particles.

Competing interests

All authors declare no competing interests in regard to any financial or non-financial relationship.

The founding institutions had no influence on study design, data collection, analysis, interpretation, writing report and decision to submit the paper for publication.

Authors' contributions

MO, EW and SdS were responsible for the concept drafting, full proposal development and getting approval from the ethics committees. MO was responsible for the specimens selection and data file cleaning. JK and WQ were responsible for the HPV DNA detection and quality control analysis. MO, EW, SdS wrote the manuscript and EW and MO were responsible for the preparation of the manuscript for submission. XFB planned the international collaborative project on HPV in invasive cervical cancer, from which Uganda is one collaborative centre.

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BMC Research Notes

SHORT REPORT Open Access

The usefulness of immunohistochemistry in tissue microarrays of Human Papillomavirus negative adenocarcinoma of the uterine cervix

Michael Odida[1,2], Belen Lloveras[3,4], Nuria Guimera[3], Elisabete Weiderpass[1,5,6,7*]

Abstract

Background: The origin of adenocarcinomas presenting on the cervix uteri may be doubtful, i.e. whether it is of cervical or endometrial origin, due to the overlapping morphological features. In HPV negative samples, further tests may be needed to ascertain the nature of the tumours. We aimed to explore the use of immunohistochemistry profiles in tissue microarrays in archived samples of adenocarcinoma (ADC) of the cervix from Uganda that tested negative for HPV DNA.

Findings: Five commercially available antibodies were tested in tissue array sections immunostained utilizing the avidin-biotin (AB) technique. In 26 ADC samples, HPV was detected in 13, p16 in 15 (8 in HPV positive and 7 in HPV negative), CEA in 12, vimentin in 6, ER in 0, and PR in 2. Among the 13/25 HPV negative ADC samples, five were positive for CEA suggesting endocervical origin, and three were vimentin positive (one had a mucinous endocervical histological pattern and two were ADC, not otherwise specified, most likely of endometrial origin).

Conclusions: The immunoprofiles of ADC with the antibodies studied are rather nonspecific. By using immunohistochemistry in 13 HPV negative ADC, endocervical tumour origin was suspected in five CEA positive cases while two out of three vimentin positive samples were probably of endometrial origin, suggesting that CEA and vimentin may be valuable in distinguishing HPV negative cervical adenocarcinomas from endometrial adenocarcinomas.

Background

Although the role of HPV in cervical adenocarcinoma appears well established [1-3], the nature of adenocarcinoma arising from the cervix may be difficult to define due to the overlapping morphological features between cervical adenocarcinomas and endometrial adenocarcinomas. While endocervical adenocarcinomas express p16 and CEA [4] and whereas endometrial adenocarcinomas usually showed vimentin and hormonal receptors [5], the results may be inconclusive. However, CEA has been reported to be expressed in both cervical and endometrial adenocarcinomas [6], while the expression of vimentin was noted to be weak and focal in many endometrial adenocarcinomas [5]. An alternative view is that some expression of these markers may reflect differentiation (mucinous versus endometrioid) compared to the histogenetic site of origin (endometrial versus cervical) [7]. In order to resolve the problem, HPV testing has been used in attempts to distinguish these tumours [8]. Extending this approach, a recent study evaluated the use of HPV DNA, p16 and hormonal status to determine the origin of cervical tumours [9].

HPV negative adenocarcinomas in the same site may originate either from cervical or endometrial cells. We postulated that immunohistochemistry could be of use in HPV negative cervical tumours from Uganda.

We aimed to explore the utility of immunohistochemistry profiles, i.e. the expression of p16, carcinoembryonic antigen (CEA), vimentin, estrogen receptor alpha (ER) and progesterone receptor (PR), in tissue microarrays in archived samples of ADC that had been tested for HPV DNA, and in particular to assess if it would be possible to define the tissue of origin of HPV negative adenocarcinoma samples. This was based on the use of CEA as a marker of endocervical ADC, vimentin and

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hormone receptors (markers more related to endometrial ADC) and p16 as a proxy marker for HPV infection. p16 is a protein encoded by p16INK4a gene and has been used as an indirect assay for HPV infection [10]. CEA is an onco-fetal protein which has been touted as a useful antibody in distinguishing between endocervical adenocarcinoma and endometrial adenocarcinoma [6]. Vimentin is characteristically positive in endometrial adenocarcinoma [5], although positivity has also been reported in cervical adenocarcinomas [6]. ER and PR are usually positive in endometrial adenocarcinoma and negative in endocervical adenocarcinoma [4]. They are used usually to exclude endocervical origin of tumours.

Materials and methods

Cervical carcinoma samples were retrieved from the archives of the Department of Pathology, Makerere University, Kampala, Uganda, and were diagnosed during the period 1968-1990. The study protocol has been approved of Higher Degrees Research & Ethics Committee at Makerere University, Uganda.

HPV testing

The cervical carcinoma samples sections selected for HPV testing were digested with proteinase K and the resulting extract was used for PCR. SPF10 PCR was performed using 10 μl of a 1:10 dilution of the DNA extract in a final reaction volume of 50 μl. The amplified PCR products were tested using probe hybridization with a cocktail of conservative probes recognizing at least 54 mucosal HPV genotypes in a microtiter plate format for the detection of HPV DNA. Optical densities (OD450) were read on a microtiter plate reader. HPV DNA positive samples were subsequently analysed by HPV SPF10-LIPA25 (version 1: produced at Labo Biomedical Products, Rijswijk, The Netherlands) [10,11], a reverse hybridization technique that detects 25 high-risk and low-risk HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74). The sequence variation within the SPF10 primers allows the recognition of these different HPV genotypes, except for the types 68 and 73 as their interprimer regions are identical and cannot be distinguished on this test. After PCR, 10 μl of the amplimers was used to perform reverse hybridization for HPV genotype identification. The positive hybridization on the strips is visualized as a purple band by means of a precipitating colour substrate on the probe site. All SPF10-LIPA25 PCR detection and typing was performed at the facilities of DDL Diagnostic Laboratories (DDL, Voorburg, Netherlands) and at Institut Català d'Oncologia (ICO, Barcelona). Detailed HPV results were published elsewhere [12].

Tissue Microarrays (TMA)

Three punches (gauge 1 mm) of pre-existing paraffin embedded tissues were obtained from each block and then re-embedded in an arrayed master block using the manual tissue microarrayer (Beecher Instruments, Silver Spring MD). The punch specimens were performed in the most representative areas of the tumours, discarding necrosis or artefacts. Four micron paraffin sections were cut from the TMA blocks and deparaffinised through alcohols and xylene before immunostaining.

Immunohistochemistry (IHC)

Monoclonal antibodies included: CEA (clone B0194-11M-P, BioGenex), p16 INK4a, p16 (clone JC8, Biocare Medical), Vimentin (clone V9, Dako), estrogen receptor alpha, ER (clone 1D5, Dako), progesterone receptor, PR (clone PgR 636, Dako).

Heat Induced Epitope Retrieval (HIER) was done using a pressure cooker as a heating device. Retrieval solutions that were used were: Citrate buffer for Vimentin, PR, ER; EDTA 10% for p16 and Saponin for CEA. Incubation was done at room temperature for 30 min for all the antibodies.

The Dako Autostainer universal staining system was used with the EnVision+ Dual Link System-HRP, a two-step IHC staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies.

The final reaction was done with diaminobenzydine and slides were counterstained with hematoxylin. The immunohistochemical stains were interpreted by one of the authors (BL) and the results were reported as positive or negative using the following criteria for each antibody. ER and PR were considered positive when more than 10% of the tumour cells displayed nuclear positive staining. p16 was considered positive/overexpressed if more than 75% of the tumour cells showed cytoplasmic and/or nuclear staining, CEA was considered as positive if more than 10% of the neoplastic cells showed cytoplasmic positive staining, and vimentin was considered positive only when more than 20% tumour cells displayed cytoplasmic staining. The cut-offs for most antibodies were those used at laboratory in Barcelona, while the 75% for p16 was chosen because this cut-off is both sensitive and specific for HPV related cervical adenocarcinoma [13].

Statistical analysis

The chi-square test was used to determine significant differences between HPV positive and HPV negative adenocarcinomas and expression of, p16, CEA, ER, PR and vimentin.

Results

In total 26 samples were successfully analysed for HPV and immunohistochemistry. In these, HPV DNA was detected in 13/26 (50%) of ADC (Table 1). Overexpression of p16 was detected in 15/26 (57.7%) ADC. Of the 15 p16 positive cases, 8 (53.3%) were HPV positive and 7 (46.7%) were HPV negative. The reactivity was both cytoplasmic and nuclear in positive samples.

CEA was positive in 12/25 (48%) ADC (Table 1). Of these, seven were HPV positive and five HPV negative. All five HPV negative and CEA positive had histological features of endocervical ADC, suggesting endocervical origin (Table 2). Vimentin was positive in 6/26 (23.1%) ADC, three of them being HPV positive. Of the three HPV negative cases, one had a mucinous endocervical histological pattern and two were ADC not otherwise specified (NOS), most likely of endometrial origin. ER was not detected in any sample and PR were positive in two samples, both HPV positive (Tables 2 and 3). Seven ADC samples were negative for all the three markers, of these two were HPV positive. Among the other five samples, two had endocervical histological features, two were serous, and one was ADC NOS. (Table 2). The positivity for each of the immunohistochemistry markers did not differ in HPV positive and negative ADC (Table 3, Figure 1).

Table 1 HPV positivity and immunohistochemistry markers in cervical adenocarcinomas (ADC)

	Adenocarcinoma (ADC)
HPV positivity	48.1% (13/26)
p16 ≥ 75%	57.7% (15/26)
CEA > 10%	48.0% (12/25)
Vimentin ≥ 20%	23.1% (6/26)
ER ≥ 10%	0.0% (0/26)
PR ≥ 10%	7.7% (2/26)

Table 3 Immunohistochemical stains by histological subtype of cervical adenocarcinoma*

	HPV +	HPV-	p value
p16 (+)	8/12	7/13	0.69
CEA (+)	7/12	5/13	0.53
Vimentin (+)	3/12	3/13	1.0
PR (+)	2/12	0/13	0.22
ER (+)	0/12	0/13	-

*Out of the 26 cases, histological sections stained with all the antibodies were available in 25.

Discussion

Histological subtypes of endocervical and endometrial ADC have some overlapping features, and poorly differentiated tumours or small biopsies are difficult to classify.

The differential diagnosis between endocervical and endometrial ADC is a common problem in surgical pathology, that can be approached using different immunohistochemical antibodies, the most accepted panel markers being CEA (as a marker of endocervical ADC), vimentin and hormone receptors (markers more related to endometrial ADC). HPV DNA detection by molecular techniques has proved valuable [8]. However in our series we found a much higher percentage of HPV negative ADC (50%) than we expected.

As a comparison, 85% of samples were positive in the whole series of the RIS TT study, which used similar HPV testing methods [14]. This could probably be explained by poor sample processing conditions in Uganda, including problems in sample transport, type of fixative (inconsistent use of buffered or non-buffered formalin) and duration of fixation, leading to poor DNA and protein preservation and consequently immunohistochemistry and PCR with equivocal results.

Overexpression of p16 has been used as a surrogate marker of high risk HPV infection [10]. Only 57.7% of

Table 2 Immunohistochemistry patterns by HPV positivity in archival adenocarcinoma samples from Uganda*

HPV positive (N = 12/25)					HPV negative (N = 13/25)	
CEA	*Vimentin*	*ER*	*PR*	% with this pattern	% with this pattern	Histological subtype
+	-	-	-	58.3% (7/12)	38.5% (5/13)	Mucinous Intestinal (2) Mucinous Endocervical (1) Mucinous NOS (1) NOS (1)
-	+	-	+	16.7% (2/12)	0.0% (0/13)	-
-	+	-	-	8.3% (1/12)	23.1% (3/13)	Mucinous Endocervical (1) NOS (2; probable endometrial)[†]
-	-	-	-	16.7% (2/12)	38.5% (5/13)	Mucinous Endocervical (1) Mucinous Villoglandular (1) NOS (1)[†,§] Serous (2; probable endometrial)[†]

*Out of the 26 samples 25 were stained with all the antibodies and HPV tested.

[†]After immunohistochemistry and histological subtype diagnosis an endometrial origin cannot be discarded.

[§]No further classification because of lack of markers.

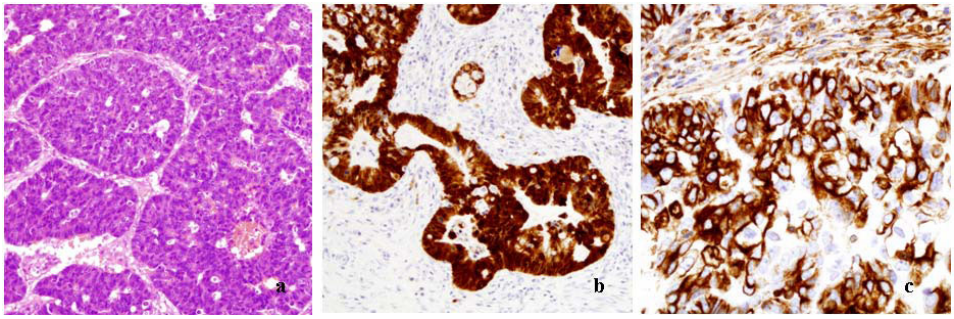

Figure 1 Adenocarcinoma. Hematoxylin-eosin (a), p16 positive staining (b) and vimentin positive staining (c).

our ADC samples were p16 positive; considering the probable rate of false negative of 15% (the rate of false negative in SCC) we should have about at least 25% (i.e. 57.7% + 15% = about 75%; 100% - 75% = 25%) of p16 negative ADC samples that remain unexplained. Being a usual problem in diagnosis, the possibility of endometrial cancer has to be considered. Moreover, p16 itself cannot be considered an ideal marker of HPV infection in ADC, since many samples of HPV negative cervical ADC have also been found to express p16 [15]. This raises the possibility of HPV-independent mechanisms of p16 overexpression in some cervical ADC, similar to what has been observed in Bowen's disease [16]. Besides, some endometrial cancer can also display overexpression of this marker [5]. Among the 13 HPV negative ADC, there were some features that support endocervical origin, i.e. CEA expression in five cases and the histological features were in favour of endocervix in three others although IHC results were inconclusive. Other studies have also found that CEA is usually positive in cervical ADC, although some samples may be negative [4,17,18]. Three of our samples showed vimentin expression which is usually related to endometrial origin [5], however one of them displayed a morphological pattern suggestive of endocervical differentiation. The expression of vimentin in cervical ADC has been reported previously [6]. The absence of ER and low expression of PR is not surprising, considering that few samples of cervical ADC are usually ER or PR positive [4].

Thus, endocervical origin seems confirmed in some samples, endometrial cancer were suspected in four others (two vimentin positive and two without conclusive IHC results but were serous ADC) and one could not be further classified due to lack of histological or IHC markers.

In view of overlapping and diverge morphologic heterogeneity of both cervical and endometrial adenocarcinomas, the results of this study suggest that the use of a number of markers appear useful in distinguishing the two tumours in HPV negative cases. In a clinical setting, one could use a combination of p16, CEA and vimentin in cervical tumours to determine possible site of origin. Another relevance of our results is in correct classification of cervical tumours for epidemiological research.

Our study has a number of strengths: only areas without necrosis were sampled, the use of tissue microarray techniques allowed uniform conditions during immunohistochemical staining, making the results more comparable, and all specimens were tested for HPV using sensitive methods.

Our study also has some limitations, such as the small number of samples studied, the probable variation in the fixation of the tissues due to the fact that samples came from different areas of Uganda, leading to loss of antigen epitopes. In particular, estrogen and progesterone receptors are known to be quite labile antigens.

Conclusion

In summary, our results showed overlap in expression of p16, CEA and vimentin between HPV positive and HPV negative cervical ADC, and suggest that some samples among the HPV negative ADC, diagnosed as cervical ADC, may be of endometrial origin. In our series amongst 13 HPV negative samples, the endocervical origin seems probable in five CEA positive samples while two vimentin positive samples are suspected to be endometrial cancer. Although the number of samples studied are too few to draw definite conclusions, it suggests that CEA and vimentin may be of value in distinguishing HPV negative cervical tissue from endometrial ADC.

List of abbreviations

ADC: adenocarcinoma; CEA: carcinoembryonic antigen; ER: estrogen receptor; HIER: Heat Induced Epitope

Retrieval; HPV: human papillomavirus; IHC: immunohistochemistry; NOS: not otherwise specified; p16: cyclin dependend kinase inhibitor 2A: tumour suppressor protein; PR: progestin receptor; PRC: polymerase chain reaction; SCC: squamous cell carcinoma; TMA: tissue microarrays.

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Authors' contributions

EW designed the study. MO had responsibility of collection of samples and designing of the database. NG conducted laboratory analysis, while BL contributed to the project with pathological expertise. MO and EW drafted the manuscript, and all authors revised the following versions of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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III

HPV types, HIV and invasive cervical carcinoma risk in Kampala, Uganda: a case-control study

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Abstract

Background: While the association of HPV with cervical carcinoma is well established, the influence of HIV on the risk of cervical carcinoma in sub Saharan Africa remains unclear. To assess the risk of invasive cervical carcinoma associated with HIV and HPV types, a hospital-based case-control study was performed between September 2004 and December 2006 in Kampala, Uganda. Incident cases of histologically confirmed invasive cervical carcinoma (N = 316) and control women (N = 314), who were visitors or care-takers of cervical carcinoma patients in the hospital, answered a standardized questionnaire including demographic, socioeconomic data, sexual behaviour patterns, medical and obstetrical history. Blood samples were obtained for HIV serology and CD4 counts. Cervical samples were obtained using a cytobrush (control women) or cervical biopsy (invasive cervical cancer women). HPV DNA detection and genotyping was performed using the SPF-$_{10}$/DEIA/LiPA$_{25}$ technique, which detects all anogenital HPVs by DEIA and identifies 25 HPV genotypes by LiPA version1. Samples that could not be typed with any of the 25 genotypes were designated as HPV X. Odds ratios (OR) and 95% confidence intervals (CI) were calculated by logistic regression, adjusting for possible confounding factors.

Results: For both squamous cell cervical carcinoma (SCC) and adenocarcinomas, statistically significantly increased ORs were found among women infected with any HPV, in particular single HPV infections, infections with HPV16- related types and high-risk HPV- related types, in particular HPV16, 18 and 45. For other HPV types the ORs for both squamous cervical carcinoma and adenocarcinomas were not statistically

significantly elevated. There was no overall association between HIV infection and risk of invasive cervical squamous cell carcinoma or adenocarcinoma after adjustment for CD4 decrease and HPV infections.

Conclusions: The OR for squamous cervical carcinoma and adenocarcinomas were increased in women infected with HPV, in particular single HPV infections, infections with HPV16- and 18- related types, infections with high-risk HPV types, specifically with HPV types 16, 18 and 45. Cervical cancer was not statistically associated with HIV infection per se, and HIV positive women did not have a higher OR for cervical cancer in relation to HPV types.

Background

Human papillomavirus infection (HPV) is a necessary cause for the development of cervical carcinoma, the risk differing [1] according to HPV type of infection. Infection with the human immunodeficiency virus (HIV) has also been associated with the risk for cervical carcinoma [2], the risk apparently varying according to co-infection with different HPV types [3,4]. The association between HIV and cervical cancer appears to be less evident in low income countries, notably in Sub-Saharan Africa, than in high income countries [5,6]. Two studies which were conducted at the beginning of the AIDS epidemic from Uganda [7] and Tanzania [8] which assessed HIV and cervical carcinoma, showed no association. However, positive association has been observed in Western countries such as Italy, France, Spain [5,9,10] as well as in some recent studies from Uganda [11] and Tanzania [12]. Whether these differences in cervical cancer risks between studies can be explained by differences in co-infection with specific HPV types remains unclear.

We present here results from a study of invasive cervical cancer in Uganda in relation to HPV and HIV infections, where the estimated world-standardized incidence rates of cervical cancer is rising and is now 52.4 per 100 000 women [13].

Methods

We conducted a hospital based case-control study in Mulago Hospital in Kampala, Uganda, which is the national referral and teaching hospital for Makerere University. The hospital admits about 10 cases with invasive cervical cancer each month.
Patients attending the Gynaecological clinics or emergency section are mostly residents of Kampala City and the surrounding areas, with a population of about 1 000 000, although some come directly from areas outside Kampala. Like most urban areas of Uganda, HIV prevalence is relatively high in Kampala [14]. Recruitment of cases and controls was done by selected nurses and midwives working in the gynaecological wards or clinics using the inclusion and exclusion criteria described below.

Recruitment of invasive cervical cancer cases

Patients eligible for the study were women aged 18 to 74 years, residents of Uganda for at least two years, consecutively diagnosed with incident invasive cervical carcinoma during the period September 2004 and September 2006. They had not yet undergone primary treatment for cervical cancer, and they gave an informed consent to participate in the study and were able to provide biological samples. Patients were excluded if tissue and blood samples could not be collected because they were in a terminal stage or for any other reason that might interfere with established patterns of patient care.

Recruitment of control women

At the Mulago Hospital, all hospitalised patients have with them one or more accompanying persons who are responsible for preparing food and taking care of the basic needs of hygiene and other requirements of the patients. These accompanying persons are in general female members of the same family or clan. Control women were recruited among those accompanying or visiting cervical cancer patients. The motivation to enrol relatives of the cancer patients as controls was to avoid bias by inclusion of control women who would most probably live in the same areas of the country as the cancer cases and would have a similar social background. They would probably also have used the same hospital as the cases if they had had a cervical cancer diagnosis. Since the evaluation of family history of cervical cancer was not among our study hypothesis, the inclusion of blood relatives of the cervical cancer patients did not constitute a problem. Control women were frequency matched to cervical cancer cases by 5-year age intervals. Inclusion criteria were the same as for cases (i.e. resident of Uganda for at least two years, aged 18 to 74 years between September 2004 and December 2006, ability to give informed consent and willing to provide biological samples), except for the cervical cancer diagnosis.

Control women were offered a pelvic examination with visual inspection of the cervix uteri and Pap smear screening test. The results were made available before they left the hospital (i.e. during the case patient period of hospitalization). Women in whom pre-malignant and malignant cervical abnormalities were suspected or detected were referred for a standard follow-up diagnosis and treatment at the Mulago Hospital. A symptomatic diagnosis of cervical and vaginal infections was done by the gynecologists examining

control women, and appropriate antibiotic treatment was offered free of charge whenever indicated.

Collection of biological samples and study procedures

Information about reproductive history, lifestyle and sexual practices was assessed using a standardized questionnaire administrated by the nurses or midwives recruiting cervical cancer cases and control women.

All subjects underwent pelvic and abdominal examinations. Cervical samples were obtained for diagnosis and HPV detection. Blood was obtained for full blood count, HIV serological testing, and CD4 counts.

HIV testing and CD4 counts

HIV was measured using a rapid test [15] which was the recommended test in Uganda during the study period (Ministry of Health of Uganda). Briefly, blood was initially tested by the Capillus method (Capillus HIV-1/HIV-2 (CP) (Trinity Biotech, Galway, Ireland). Negative results were reported as negative, and no other confirmatory test was done. For positive results, a confirmatory test using Serocard (Trinity Biotech, Galway, Ireland) was performed. When results from Serocard were positive, it was reported as positive. However, if the results from Serocard were negative, a tiebreaker test using the Multispot test (Bio-Rad Laboratories) was applied, and the result reported as negative or positive, depending on the result of this test. All tests were done according to the manufacturer's instructions. HIV was assessed as positive (exposed) or negative (not exposed). These tests algorithms have 100% sensitivity and specificity [15]. The CD4 counts were done

using flow cytometry with the Becton Dickinson FACSCount automated instrument (BD FACSCount™).

Cervical cell samples

For women with invasive cervical carcinoma, biopsies of the cervical lesions were collected under general anesthesia. Samples were immediately stored into sterile specimen tubes and transported in a flask containing ice to the pathology laboratory. There the specimen was divided into two parts: one part was fixed in 10% buffered formalin (for 12 to 24 hours) for histological processing and diagnosis, and the other half was kept in a Nuc tube, labelled and stored at -70^{O}C until shipped for HPV analysis. Biopsy samples were processed using the automatic tissue processor; they were paraffin embedded, sectioned at 4 µm thickness and stained with Haematoxylin and Eosin.

Exfoliated cervical cells were collected before any treatment from control women with two Cervex brushes. The first brush was rinsed in PreservCyt solution (ThinPrep, Cytyc Corporation) according to the manufacturer's instructions and kept at room temperature until shipped to the laboratory for HPV testing. The second brush was used to make a smear and stained with Pap stain; smears were classified as normal or abnormal.

All histological and cytological diagnoses were done by a pathologist at the Department of Pathology, Makerere University.

HPV testing

The frozen invasive cervical carcinoma samples and the exfoliated cervical samples (inThinPrep) from control women were shipped to Delft Diagonstic Laboratory (DDL) in

Voorburg, the Netherlands, for HPV analysis. Frozen samples were shipped in dry ice, while Thin Prep samples were shipped at room temperature. DNA was extracted from the frozen sections of cervical tissue samples using proteinase K. From the suspension of cervical cells, DNA was isolated by use of a MagNA Pure LC instrument (Roche Diagnostics) using the total nucleic acid isolation kit (Roche Diagnostics).

SPF_{10} PCR was performed using 10 µl isolated DNA in a final reaction volume of 50µl. Ten µl of the amplified PCR products were tested with a cocktail of general probes recognizing at least 54 mucosal HPV genotypes in a microtiter plate format for the detection of HPV DNA by use of DNA enzyme immunoassay (DEIA). Optical densities (OD_{450}) were read on a microtiter plate reader. Subsequently, for HPV DNA positive samples 10µl of the same SPF_{10} amplimer was genotyped by the SPF10-$LiPA_{25}$ test (version 1, produced at Labo Biomedical Products, Rijswijk, The Netherlands). By reverse hybridization of the SPF_{10} amplimer with 28 different probes on the LiPA strip 25 HPV genotypes can be identified: HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, 74). The sequence variation within the SPF_{10} primers allows the recognition of these different HPV genotypes, except for the types 68 and 73, as their innerprimer regions are identical and cannot be distinguished in this test. The positive hybridization on the strips is visualized as a purple band by means of a precipitating colour substrate on the probe site. Samples that were HPV DNA positive with SPF_{10} PCR-DEIA, but for which no type was identified by the $LiPA_{25}$ strip were designated as HPV type X.

For invasive cervical cancer cases that were HPV negative, a beta-globin PCR was performed to assess the quality of DNA isolated from the biopsy tissue samples tested negative for HPV DNA.

Ethical consideration

The study protocol was approved by the Higher Degree's Research Committee of the Faculty of Medicine, Makerere University and the Uganda National Council of Science and Technology. The study also complied with the ethical norms of Mulago Hospital and those of the Ministry of Health of Uganda. All the study participants gave a written informed consent before inclusion into the study.

Data analysis

To evaluate the risk of cancer, SCC and adenocarcinoma separately, odds ratios (OR) and the associated two-sided 95% confidence intervals (CI) were calculated using logistic regression models. To address the problems of small sample sizes, we calculated the logistic regression using Firth's penalized likelihood approach [16]. As a consequence of the small sample sizes, only the lower or upper bound of the confidence interval was possible to estimate for some ORs. We fitted age-adjusted models for each HPV type or groups of HPV types, as well as models also adjusting for HIV (positive or negative) and CD4 count per 100 cells, as a linear continuous variable(s).

The combined groups of HPV types analyzed were "any HPV infections"; "single HPV infections" for any HPV infection except HPVX, "multiple HPV infections", i.e. concomitant infection with more than one HPV type , except for HPVX, "high-risk HPV"

for infections with any of the HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 or 73; ”low-risk HPV” for infections with any of the HPV types 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70 or 74; ”HPV16- related” for any of the HPV types 16, 31, 33, 35, 52 or 58; ”HPV18- related” for any of the types 18, 39, 45, 59 or 68; and HPVX.

The statistical software SAS version 9.2 procedure logistic was used for all analyses. All tests of statistical hypotheses were made on the two-sided 5% level of significance.

Results

During the study period, 484 women with a preliminary incident cervical carcinoma diagnosis and 385 control women were approached and invited to participate in the study. Sixty six (66) potential cervical carcinoma cases and 22 control women declined to participate, and 41 cases and 44 control women were excluded due to incomplete questionnaires or inability to provide any samples for different reasons. Further, 61 potential cases were excluded because the diagnosis of invasive cervical cancer was not confirmed by histology, as well as 5 control women who had cervical lesions compatible with cervical intraepithelial neoplasia (CIN). Thus, a total of 316 invasive cervical cancer cases (265 squamous cell carcinomas, 42 adenocarcinomas, 5 of mixed histology and 4 other histology) and 314 control women were preliminary recruited into the study. From these, cervical samples for HPV testing were obtained in 255 invasive cervival cancer cases (215 were squamous cell carcinomas, 32 adenocarcinomas and eight (8) were other histological types) and 309 controls. Of 255 invasive cervical cancer cases tested for HPV, 16 had to be excluded because their samples were beta-globin negative, leaving 239 cases in the final analysis. The details of the enrolment are shown in Table 1.

The clinical and demographic characteristics of study participants are shown in Table 2. A smaller proportion of cases than controls had ever had a Pap smear taken, and cases had proportionally more HPV infections than controls.

Of the 239 cases and 309 controls, HPV genotypes were detected in 222 (92.9% of 239) of cases and 95 (30.7% of 309) of control women. Seventeen cases (7.1%) were HPV negative.

There were no other statistically significantly differences between cases and controls regarding characteristics, including HIV positivity. The HIV test was positive in 55 (18.8%) cervical cancer cases and 54 (17.5%) control women.

Squamous cell carcinoma (SCC) and HPV

For SCC, the age-adjusted ORs were statistically significantly increased for any HPV positive (OR 50.8, 95% CI: 25.8-113.9), single HPV infections (OR 33.3, 95% CI: 20.2-56.7), HPV16- related types (OR 11.6, 95% CI: 7.5-18.5), HPV18- related types (OR 9.4, 95% CI: 5.3-17.4), and high-risk HPV types (OR 64.5, 95% CI: 35.4-126.6), in particular HPV16 (OR 29.1, 95% CI: 15.4-60.7), HPV18 (OR 9.6, 95% CI: 4.6-22.3), and HPV45 (OR 58.7, 95% CI: lower limit >7.9).

Multiple HPV infections and infections with low-risk HPV types were not associated with increased an OR for SCC. Adjustment for HIV as well as CD4 count decline did not change substantially the OR estimates with the different HPV types (Table 3).

Squamous cell carcinoma according to HPV and HIV

The association between different types of HPV infections and SCC was rather similar in women who were HIV negative and HIV positive, although the number of HIV positive SCC cases in our study (N = 42) was limited, and therefore the confidence intervals of our OR estimates were rather broad (Table 4).

Adenocarcinoma and HPV

The OR for adenocarcinoma were statistically significantly increased for any HPV positive (OR 12.9, 95% CI: 5.0-41.8), single HPV infections (OR 13.7, 95% CI: 6.0-34.0), HPV16- related infections (OR 3.6, 95% CI: 1.5-8.2), HPV18- related infections (OR 22.8, 95% CI: 9.3-58.9), high-risk HPV infections, in particular with HPV 16 (OR 11.6, 95% CI: 4.3-31.8), 18 (OR 18.5, 95% CI: 6.6-54.4) and 45 (OR 297.4, 95% CI: lower limit >26.4). Adjustment for HIV as well as CD4 count decline did not change substantially the OR for the different HPV types (Table 5).

Adenocarcinoma according to HPV and HIV infection

Given the very low number of HIV positive adenocarcinoma cases (N = 4) in our study, our risk estimates are not sufficiently precise to reach any conclusions about differences between HIV negative and positive women, but there was no suggestion of a risk difference (Table 6).

Table 7 shows the overall OR for SCC and adenocarcinoma in relation to HIV infection. In statistical models adjusting for age and CD4 count, HIV positive women had an increased OR for SCC (OR 1.6, 95% CI: 1.0-2.6). However, after further adjusting for different HPV infections, the OR became no longer statistically significant (adjustment for age, CD4 and any HPV infection: OR 0.8, 95% CI: 0.4-1.4), except when we only adjusted for low-risk HPV infections (OR 1.9, 95% CI: 1.1-3.2) and multiple infections (OR 1.8, 95% CI: 1.1-3.0), when the lower confidence intervals were of borderline statistical significance. There was no evidence of association between HIV infection and adenocarcinoma in any of the statistical models tested.

Discussion

The HPV types associated with both SCC and adenocarcinoma risk in our study has also been found to be associated with the most invasive cervical carcinoma in other regions of the World [17,18].

Squamous cell carcinoma

Our study shows that SCC in Uganda is strongly associated with any HPV infections and high-risk HPV infections, in particular high-risk infections with HPV16 (and related types), 18 (and related types) and 45. We found no evidence that adjustment for HIV infection or CD4 decline affect the OR significantly, or that HIV infection per se was associated with SCC.

As in our study, several earlier studies found HPV16 predominating in squamous cell carcinomas [19]. Our study showed higher ORs for SCC associated with HPV16 (OR 29.1, 95% CI: 15.4-60.7), which is similar to a study from Taiwan by Chen et al. [20], where the HPV16 was associated with an OR of 67. The Taiwanese study found substantial elevated risks for SCC associated with HPV52 (OR = 3.04) and HPV58 (OR = 5.22), which were not confirmed in our study, while the risks for SCC with the other HPVs were low. A study from Mexico also found a high risk of cervical carcinoma for HPV16 and 18 [1]. The results from Mexico are exceptional in that the OR for HPV18 was higher than that of HPV16. High risks for HPV18 have also been previously reported by a number of authors [21-23]. Low risks for HPV18 have been found in some studies from India [24,25].

Adenocarcinomas

For adenocarcinomas, we found an association between infections with any HPV infections, in particular single infections, infections with high-risk HPV types, most importantly HPV16 (and related types), 18 (and related types) and 45. Adjustment for HIV infection and CD4 decline did not alter the associations meaningfully, suggesting the little confounding effect of HIV and its associated immune dysfunction on the risk of HPV in cervical carcinoma. The number of HIV positive cases in our study was rather small, and therefore no firm conclusion could be reached regarding any differences in the association between HPV infections in HIV positive and negative women.

HPV18 has been reported as the most frequent HPV type found in adenocarcinomas [19]. In a pooled analysis of case-control studies, the relative risk of HPV18 in cervical adenocarcinoma was high (OR 410.32, 95% CI: 167.44 to ∞) [4]. These results were much higher then ours for HPV18 (OR 18.5, 95% CI: 6.6-54.4).

These differences in findings in different studies may indicate that regional factors, such as differences in the prevalence of different types of HPV infections, may affect cervical carcinoma risks [26]. These differential risks are probably due to pathogenesis of the two major types of cervical carcinoma [27]. Smith et al. [28] and Madeleine et al. [29] noted an elevated risk of SCC associated with trichomonas, but no associated increased risk for adenocarcinoma. Another factor which could in theory explain the differences in risks of squamous cell carcinoma and adenocarcinoma is smoking [30]; however smoking is not common in Uganda with a prevalence of 3% among adult women [31] and 5.3% among adolescent girls [32]. The relatively low relative risk of cervical carcinoma due to other

high-risk HPV types (besides HPV16, 18 and 45) is supported by the low prevalence of these HPV types in cervical carcinomas in several other studies [3,33], including a recent study by de Cremoux et al. [34] who found that up to one third of cervical carcinomas were not associated with HPV16 or 18. Some of these high-risk types, namely HPV31, 33, 52 and 58 have been found to be more prevalent in high grade intraepithelial cervical lesions than in invasive squamous cell carcinomas [17].

HIV

Of particular interest was the lack of effect of HIV infection and associated reduction of CD4 count on the risk for cervical carcinomas. After controlling for HPV infections in the statistical models, the association between HIV and SCC became non-statistically significant for most HPV infections, except low-risk and multiple infections, while there was no association between HIV and adenocarcinomas, regardless of which statistical adjustment we did in the models.

The lack of excess increased risk of cervical carcinoma as a result of HIV infection could be explained by the fact that progression of intraepithelial neoplasia to invasive carcinoma are multifactorial, and not dependent on immune status only [35], and the existence of slow progressors [36,37] or elite controllers [38,39] in some women. Lastly, infections with HIV may have occurred too late in life to have any effect on HPV infections, as suggested by a study from Nairobi, Kenya [40].

Several previous studies have shown varying risk of cervical carcinoma in association with HIV infection [12,41-44]. Earlier studies suggested that a possible increase of

cervical cancer risk among HIV positive women could be attributed to the increased prevalence of HPV infection, persistence of HPV infection and pre-invasive cervical carcinoma [45]. In addition, CIN is less likely to regress in HIV-infected women [46]. This has been attributed to reduced HLA class II molecules and the presence of immature Langerhans cells within the cervical tissue in HIV-infected women [47]. However, our study did not corroborate the hypothesis that HIV infection would increase a woman's risk for invasive cervical cancer, and the OR associated with different HPV infections and cervical cancer risk were similar in HIV positive and negative women, except for low-risk and multiple infections for SCC. This could be attributed to the role of impaired immunity as previously suggested [45,46]. Other studies have suggested that HPV types other than 16 or 18 are more prevalent in HIV positive women [19,48], and that the median duration of infections with different individual HPV types lasts longer in HIV positive women as compared to HIV negative women, with the exception of HPV16 which is similar [49,50].

Our study had a number of advantages compared to previous studies. We enrolled all consecutive patients with lesions suspected to be invasive cervical cancer, aiming to reduce bias that could occur if any criteria associated with the exposure were used to select cases, for example stage, duration of symptoms, tribe, etc. While other studies have used women with other disease conditions as controls [11,12], we were able to use women who were not hospitalised, had no cervical disease (CIN cases were excluded), and were living in the same geographical areas as cancer cases. Further, they were representative of the population base from where the cases arose. Since the evaluation of

family history of cervical cancer was not among our study hypothesis, the inclusion of blood relatives of the cervical cancer patients as controls did not constitute a problem.

Another strength was that we carried out the study in an area where both HIV prevalence [14] and cervical carcinoma incidence are high [13]. This allowed us to adjust for HIV status and decline in CD4 counts in our HPV analysis, as well as to perform stratified analysis by HIV status to assess the impact of HIV infection per se and the HPV risk associated with HIV status on cervical carcinoma.

Some possible confounding could not be controlled, as the information was not available in our questionnaire. For example, exposure to indoor air pollution caused by smoke from cooking fire, which has been associated with cervical carcinoma [51]. Like in most developing countries, the use of firewood for cooking is highly common in Uganda. However, this would have affected both cases and controls. Another factor is the education level. However, a pooled analysis showed that there were no differences in HPV positivity by education level, and concluded that the excess of cervical cancer found in women with little education is not due to the excess of HPV prevalence, but rather by early events which may modify the cancer causing ability of HPV [52].

Our study was limited by the number of probes for HPV which could only allow us to type 25 HPV genotypes, and the relatively small number of HIV positive cases of SCC and in particular adenocarcinomas.

In addition, assessment of immunity was based only on CD4 counts, which reflects HIV infection on the systemic immune system. Although CD4 counts have been used before [53] it is difficult to know when immunodeficiency sets in. The statistical power for detecting cancer risk in subgroups of HIV-infected women was limited, in particular for adenocarcinoma.

Conclusions

In conclusion, the OR for squamous cell carcinoma and adenocarcinoma of the cervix in Uganda were increased in women infected with HPV, in particular single HPV infections, infections with HPV16- and 18-related types, infections with high-risk HPV types, specifically with HPV16, 18 and 45. Cervical cancer was not statistically associated with HIV infection per se, and HIV positive women did not have a higher OR for cervical cancer in relation to most HPV types, except for low-risk and multiple infections in SCC.

List of abbreviations

AIDS, acquired immune deficiency syndrome; BD FACSCount, automated instrument for monitoring of HIV/AIDS; CD4, cluster of differentiation 4, glycoprotein, in humans encoded by the *CD4* gene; CI, confidence intervals; CIN, cervical intraepithelial neoplasia; DDL, Delft Diagnostic Laboratory; DEIA, DNA enzyme immunoassay; DNA, deoxyribonucleic acid; HIV, human immunodeficiency virus; HPV, human papillomavirus; OR, odds ratio; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; SPF_{10}-$LiPA_{25}$, system for HPV genotyping

Competing interests

All authors declare no conflict of interest

Authors' contributions

MO, FM and EW were responsible for developing the concept, full proposal development and getting ethical approvals. MO carried out the fieldwork and was responsible for data collection. BK and WQ made all laboratory analyses and contributed to the interpretation of laboratory results. SS provided statistical analyses and interpretation of the results. MO and EW wrote the manuscript, while all other authors; FM, BK, WK and SS gave their critical comments upon the writing process and revised the final manuscript. All authors approved the version to be published.

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Tables

Table 1: Subjects' enrolment and measurements. Invasive cervical cancer study in Kampala, Uganda

	Cases	%	Controls	%
Women invited to participate	484	100.0	385	100.0
Declined to participate	66	13.6	22	5.7
Incomplete questionnaires	41	8.5	44	11.4
Diagnosis not confirmed[a]	61	12.6	5	1.3
Total preliminary recruited[b]	316	100.0	314	100.0
Samples for HPV testing[b]	239[c]	75.6	309	98.4
Samples for HIV testing[b]	281	88.9	308	98.1
Combined HIV and HPV samples[b]	222	70.3	303	96.5

[a] Diagnosis of invasive cervical cancer not confirmed by histology for cases; control women found with abnormal cervical smears (cervical intraepithelial neoplasia, CIN, not invasive cervical cancer)

[b] Percent based on total recruited

[c] In addition to these 239, 16 cases were beta-globin negative and therefort excluded from all analyses

Table 2: Clinical and demographic characteristics of cases and controls. Invasive cervical cancer study in Kampala, Uganda

	Cases[a]		Controls	
	N	% [b]	N	% [b]
Number of women	316		314	
Mean age years (SD)	47	11.5	41	12.5
Mean age first intercourse (SD)	16	3.0	16	3.0
Mean parity (SD)	6.9	5.8	4.8	4.0
Mean CD4 counts (SD)	722	375	843	389
Ever used hormonal contraceptives	103	32.6	111	35.4
HIV status (+)[c]	55	18.8	54	17.5
Had attended school	207	65.5	271	86.3
Ever smoked regularly	26	8.2	26	8.3
Partner is a current smoker	76	24.1	67	21.3
Alcohol consumption	131	41.5	133	42.4
History of sexually transmitted diseases	148	46.8	137	43.6
Ever had Pap smear	6	1.9	13	4.1
HPV tested	239	75.6	309	98.4
HPV detected	222	92.9[d]	95	30.7[d]
HPV not detected	17[e]	7.1[d]	214	69.3[d]

[a] Number of women with: squamous cell carcinoma: 265, adenocarcinoma: 42, mixed histology: 5, other: 4

[b] Percent where applicable

[c] HIV positivity based on available data for 293 cases and 308 controls

[d] Percentage (%) of tested

[e] Additional 16 invasive cervical cancer patients had available samples, but these were beta-globine negative, indicating poor sample quality, and therefore were excluded from all analyses

Table 3: Risk[a] of squamous cell carcinoma of the cervix for different HPV types

HPV type	HPV+		Age adjusted N = 203 cases & 309 controls		Age and HIV adjusted N = 193 cases & 303 controls		Age, HIV and CD4 adjusted N = 192 cases & 296 controls	
	Cases	Controls	OR	95% CI	OR	95% CI	OR	95% CI
High-risk HPV								
All[b]	183	54	64.5	35.4-126.6	84.7	43.5-181.6	82.3	42.2-176.8
16	101	10	29.1	15.4-60.7	28.8	15.2-60.2	27.2	14.4-56.9
18	39	8	9.6	4.6-22.3	8.9	4.2-20.6	8.3	4.0-19.2
31	2	3	0.8	0.1-4.6	0.7	0.1-3.7	0.5	0.1-2.8
33	4	5	1.3	0.3-4.7	1.2	0.3-4.4	1.4	0.3-5.6
35	10	7	2.4	0.9-6.7	2.5	0.9-6.9	2.7	1.0-8.1
39	3	1	3.7	0.6-38.9	4.2	0.7-44.4	4.2	0.7-44.2
45	18	0	58.7	>7.9[j]	56.5	>7.6[j]	57.2	>7.7[j]
51	2	8	0.8	0.2-2.7	0.5	0.1-2.1	0.5	0.1-2.1
52	6	13	0.7	0.2-1.7	0.6	0.2-1.5	0.5	0.2-1.3
56	2	4	0.9	0.2-4.6	1.0	0.2-4.7	1.1	0.2-5.5
58	2	3	0.9	0.2-5.1	1.0	0.2-5.8	1.1	0.2-5.8
59	1	2	0.7	0.1-6.5	0.8	0.1-7.4	0.8	0.1-7.4
68/73	6	7	1.5	0.5-4.6	1.3	0.4-4.0	1.3	0.4-3.9
Low-risk HPV								
All[c]	5	24	0.5	0.2-1.0	0.3	0.1-0.8	0.3	0.1-0.7
6	1	3	1.0	0.1-7.0	1.1	0.1-8.0	1.1	0.1-7.9
11	0	2	0.5	<6.4[k]	0.4	<5.4[k]	0.3	<4.3[k]
34	0	1	0.6	<11.1[k]	0.4	<7.5[k]	0.3	<5.3[k]
40	0	1	0.7	<13.0[k]	0.8	<14.7[k]	0.8	<14.6[k]

42	0	0	4.1	>0.2[j]	[l]	[l]	[l]	[l]
44	0	4	0.2	<2.2[k]	0.3	<2.5[k]	0.2	<2.5[k]
53	0	4	0.1	<1.2[k]	0.1	<1.2[k]	0.1	<1.1[k]
54	1	3	0.4	<2.5[k]	0.4	<2.8[k]	0.5	<2.9[k]
66	0	5	0.4	<2.2[k]	0.1	<1.0[k]	0.1	<1.1[k]
70	1	3	0.7	0.1-4.2	0.6	0.1-3.7	0.5	0.1-3.2
74	2	4	1.0	0.1-4.2	0.9	0.2-4.6	0.8	0.1-3.8
Single inf.[d]	168	49	33.3	20.2-56.7	31.5	19.1-54.0	31.0	18.7-53.5
Multiple inf.[e]	15	20	1.3	0.7-2.7	1.2	0.6-2.4	1.1	0.5-2.3
16- related[f]	123	36	11.6	7.5-18.5	11.9	7.6-19.0	11.4	7.2-18.3
18- related[g]	66	16	9.4	5.3-17.4	8.9	5.0-16.5	8.6	4.9-16.1
HPVX[h]	3	26	0.2	0.1-0.5	0.2	0.1-0.5	0.2	0.1-0.6
Any HPV[i]	186	95	50.8	25.8-113.9	55.7	27.2-132.4	54.9	26.7-130.4

[a] OR and 95% CI

[b] High-risk HPV: 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68/73

[c] Low-risk HPV: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, 74

[d] Single infection: infection with any of the HPV types above, except HPVX

[e] Multiple infections: infections with at least two different HPV types, except HPVX

[f] HPV16- related: includes HPV 16, 31, 33, 35, 52 and 58

[g] HPV18- related: includes HPV 18, 45, 59 and 68

[h] HPVX: Samples which could not be typed with any of the 25 genotypes abowe

[i] Any HPV infection: infection with any of the HPV types above

[j] Indicate only lower limit value of 95% CI

[k] Indicate only upper limit value of 95% CI

[l] Could not be estimated due to lack of cases with known HIV status

Table 4: **Squamous cell carcinoma of the cervix according to different HPV types and HIV serology**

	HIV Negative women						HIV Positive women					
	Cases		Controls				Cases		Controls			
HPV types	(N = 162)		(N = 251)				(N = 42)		(N = 52)			
	HPV		HPV				HPV		HPV			
	+	-	+	-	OR[a]	95% CI	+	-	+	-	OR[a]	95% CI
High-risk[b]	142	10	35	216	76.4	38.8-165.6	41	1	19	33	132.3	>17.0[j]
16	78	74	7	244	32.8	15.6-78.9	23	18	3	49	16.5	5.3-67.6
18	27	125	2	249	22.3	7.1-111.8	12	29	6	46	2.9	1.0-9.1
31	0	152	2	249	0.1	<1.5[i]	2	39	1	51	2.3	0.3-26.4
33	4	148	2	249	2.5	0.5-14.8	0	41	3	49	0.2	<2.7[i]
35	10	142	4	247	4.5	1.5-16.0	0	41	3	49	0.2	<2.0[i]
45	13	139	0	251	41.8	>5.4[j]	5	36	0	52	17.1	1.8-2287
Low-risk[c]	3	149	18	233	0.3	0.1-0.9	2	39	6	46	0.5	0.1-2.0
Single inf.[d]	132	20	38	213	34.0	19.4-62.5	36	5	11	41	21.4	7.5-71.0
Multiple inf.[e]	10	142	9	242	2.0	0.8-5.1	5	36	11	41	0.6	0.2-1.9
16- related[f]	98	54	22	229	17.0	10.0-30.1	25	16	14	38	3.8	1.6-9.4
18- related[g]	49	103	9	242	11.9	5.9-26.4	17	24	7	45	4.5	1.7-13.1
Any HPV[h]	145	7	66	185	51.3	24.8-122.6	41	0	29	23	60.0	>7.7[j]

[a] All odds ratios are age-adjusted

[b] High-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73

[c] Low-risk HPV: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, 74

[d] Single infection: infection with any of the HPV types above

[e] Multiple infections: infections with at least two different HPV types

[f] HPV16- related: includes HPV 16, 31, 33, 35, 52 and 58

[g] HPV18- related: includes HPV 18, 45, 59 and 68

[h] Any HPV infection: infection with any of the HPV types above

[i] Indicate only upper limit value of 95% CI

[j] Indicate only lower limit value of 95% CI

Table 5: Adenocarcinoma of the cervix for different HPV types[a]

HPV type	Number HPV+		Age adjusted		Age and HIV adjusted		Age, HIV and CD4 adjusted	
	Cases	Controls	OR	95% CI	OR	95% CI	OR	95% CI
High-risk HPV								
All[b]	26	54	27.2	10.4-88.9	40.5	14.1-157.4	40.0	13.9-155.6
16	10	10	11.6	4.3-31.8	14.5	5.2-42.6	13.8	4.9-40.2
18	10	8	18.5	6.6-54.4	26.3	8.3-96.5	24.0	7.7-86.9
31	1	3	2.2	0.2-16.1	2.6	0.2-19.6	2.6	0.2-19.2
33	0	5	1.2	<12.2[j]	1.4	<14.6[j]	1.3	<14.1[j]
35	0	7	0.8	<7.1[j]	0.9	<8.3[j]	0.8	<7.9[j]
39	0	1	5.5	<108.6[j]	4.7	<92.5[j]	4.7	<92.5[j]
45	4	0	297.4	>26.4[k]	248.3	>22.1[k]	247.8	>22.0[k]
51	2	8	3.9	0.7-16.9	4.1	0.7-17.6	4.1	0.7-17.7
52	0	13	0.3	<2.4[j]	0.4	<3.4[j]	0.3	<2.9[j]
56	0	4	0.8	<11.0	1.0	<12.1[j]	1.0	<12.8[j]
58	0	3	1.3	<15.5[j]	1.3	<14.9[j]	1.3	<14.9[j]
59	0	2	0.9	<18.6[j]	1.1	<19.27[j]	1.1	<19.8[j]
68/73	2	7	3.0	0.5-13.7	3.6	0.6-16.2	3.4	0.6-16.0
Low-risk HPV								
All[c]	1	24	0.6	0.1-2.4	0.6	0.1-2.7	0.6	0.1-2.6
6	1	3	12.8	1.1-96.7	10.0	0.9-76.0	10.0	0.9-75.9
11	0	2	3.7	<55.1[j]	4.6	<64.5[j]	3.8	<56.5[j]
34	0	1	4.3	<82.4[j]	6.4	<161[j]	4.6	<126.4[j]
40	0	1	5.3	<102.7[j]	4.5	<88.3[j]	4.5	<88.3[j]
44	1	4	5.4	0.5-35.2	4.8	0.4-30.3	4.8	0.4-30.3
53	0	4	0.7	<7.6[j]	0.8	<8.7[j]	0.8	<8.3[j]
54	0	3	0.6	<7.4[j]	0.7	<8.0[j]	0.7	<8.0[j]
66	0	5	0.9	<9.0[j]	1.2	<12.7[j]	1.0	<11.2[j]
70	0	3	1.3	<15.3[j]	1.6	<17.7[j]	1.4	<16.0[j]

74	0	4	0.8	<11.1[j]	0.9	<11.9[j]	0.9	<11.5[j]
Single inf.[d]	22	49	13.7	6.0-34.0	15.5	6.6-40.5	15.3	6.5-40.0
Multiple inf.[e]	4	20	2.5	0.7-7.5	3.3	0.9-10.5	3.1	0.8-9.8
16- related[f]	11	36	3.6	1.5-8.2	4.9	2.0-11.6	4.7	1.9-11.2
18- related[g]	16	16	22.8	9.3-58.9	26.2	10.3-71.8	24.5	9.7-67.0
HPVX[h]	0	26	0.2	<1.2[b]	0.2	<1.3[b]	0.2	<1.5[b]
Any HPV[i]	26	95	12.9	5.0-41.8	18.7	6.6-71.6	18.6	6.6-71.2

[a] Analysis conducted with 32 cases and 309 controls

[b] High-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73

[c] Low-risk HPV: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, 74

[d] Single infection: infection with any of the HPV types above, except HPVX

[e] Multiple infections: infections with at least two different HPV types, except HPVX

[f] HPV16- related: includes HPV 16, 31, 33, 35, 52 and 58

[g] HPV18- related: includes HPV 18, 45, 59 and 68

[h] HPVX: Samples which could not be typed with any of the 25 genotypes abowe

[i] Any HPV infection: infection with any of the HPV types above

[j] Indicate only upper limit value of 95% CI

[k] Indicate only lower limit value of 95% CI

Table 6: Adenocarcinoma of the cervix according to different HPV types and HIV serology

	HIV Negative women						HIV Positive women					
	Cases		Controls				Cases		Controls			
HPV types	(N = 28)		(N = 251)				(N = 4)		(N = 52)			
	HPV		HPV				HPV		HPV			
	+	-	+	-	OR[a]	95% CI	+	-	+	-	OR[a]	95% CI
High-risk[b]	24	3	53	216	39.5	13.6-153.8	2	0	19	33	8.8	0.7-1235
16	8	19	7	244	11.0	3.6-34.2	2	0	3	49	108.2	>4.0[i]
18	10	17	2	249	57.9	14.8-327.8	0	2	6	46	1.3	<20.8[j]
31	1	26	2	249	2.8	0.2-24.2	0	2	1	51	6.0	<149.8[j]
33	0	27	2	249	1.8	<23.7[j]	0	2	3	49	3.5	<71.4[j]
45	4	23	0	251	251.3	>22.2[i]	0	2	0	52	[k]	[k]
Low-risk[c]	1	26	18	233	0.7	0.1-3.2	2	0	6	46	1.4	<19.8[j]
Single inf.[d]	7	20	38	213	14.5	6.0-38.2	2	0	11	41	15.0	1.2-2086
Multiple inf.[e]	4	23	9	242	5.0	1.3-16.9	2	0	11	41	0.9	<11.0[j]
16- related[f]	9	18	22	229	4.3	1.6-10.5	2	0	14	38	12.8	1.0-1794
18- related[g]	16	11	7	244	42.2	15.0-133.8	0	2	7	45	1.3	<17.8[j]
Any HPV[h]	24	3	66	185	18.3	6.4-70.0	2	0	29	23	4.0	0.3-565.6

[a] All odds ratios are age-adjusted

[b] High-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73

[c] Low-risk HPV: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, 74

[d] Single infection: infection with any of the HPV types above

[e] Multiple infections: infections with at least two different HPV types

[f] HPV16- related: includes HPV 16, 31, 33, 35, 52 and 58

[g] HPV18- related: includes HPV 18, 45, 59 and 68

[h] Any HPV infection: infection with any of the HPV types above

[i] Indicate only lower limit value of 95% CI

[j] Indicate only upper limit value of 95% CI

[k] Could not be estimated due to lack of cases with known HIV status

Table 7: Squamous cell carcinoma and adenocarcinoma of the cervix according to particular adjustment factors

	Squamous cell carcinoma N = 246 cases, 308 controls[a]		Adenocarcinoma N = 39 cases, 308 controls[b]	
Adjustment factor	OR	95% CI	OR	95% CI
Age	1.5	0.9-2.3	1.1	0.4-2.7
Age + CD4	1.6	1.0-2.6	0.8	0.1-2.6
Age + CD4 + HPV16	1.6	0.9-2.9	0.5	0.1-1.8
Age + CD4 + HPV16-related[c]	1.4	0.7-2.4	0.5	0.1-1.6
Age + CD4 + HPV18	1.4	0.8-2.4	0.3	<1.2[j]
Age + CD4 + HPV18-related[d]	1.4	0.8-2.4	0.4	0.1-1.5
Age + CD4 + High-risk HPV[e]	0.6	0.3-1.1	0.3	<0.9[j]
Age + CD4 + Low-risk HPV[f]	1.9	1.1-3.2	0.8	0.2-2.6
Age + CD4 + Single HPV inf.[g]	1.2	0.6-2.4	0.5	0.1-1.7
Age + CD4 + Multiple HPV inf.[h]	1.8	1.1-3.0	0.6	0.1-2.0
Age + CD4 + Any HPV inf.[i]	0.8	0.4-1.4	0.4	0.1-1.2

[a] HIV positive: 48 cases, 54 controls

[b] HIV positive: 5 cases, 54 controls

[c] HPV16- related: includes HPV 16, 31, 33, 35, 52 and 58

[d] HPV18- related: includes HPV 18, 45, 59 and 68

[e] High-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73

[f] Low-risk HPV: 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, 74

[g] Single infection: infection with any of the HPV types above

[h] Multiple infections: infections with at least two different HPV types

[i] Any HPV infection: infection with any of the HPV types above

[j] Indicate only lower limit value of 95% CI

IV

Comparison of human papillomavirus detection between freshly frozen tissue and paraffin embedded tissue of invasive cervical cancer

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Abstract

Background

Human Papillomavirus (HPV) detection results comparing paraffin embedded cervical tissue and other cervical specimens have been done with varying degrees of agreement. However, studies comparing freshly frozen specimens and paraffin embedded specimens of invasive cervical carcinomas are lacking. The aim of the study was to compare HPV detection using SPF_{10} broad-spectrum primers PCR followed by DEIA and genotyping by $LiPA_{25}$ (version 1) between freshly frozen cervical tissue samples and paraffin embedded blocks of cervical tissue from the same patient. There were 171 pairs of paraffin embedded and freshly frozen samples analyzed from cervical carcinoma cases from Kampala, Uganda.

Results

88.9% (95% CI: 83.2%-93.2%) of paraffin embedded samples were HPV positive compared with 90.1% (95% CI: 84.6%-94.1%) of freshly frozen samples, giving an overall agreement in HPV detection between fresh tissue and paraffin embedded tissue at 86.0% (95% CI: 79.8%-90.8%). Although the proportion of HPV positive cases in freshly frozen tissue was higher than those in paraffin blocks, the difference was not statistically significant ($p > 0.05$). In both types of tissues, single HPV infections were predominant, with HPV16 accounting for 47% of positive cases. Comparison in the overall agreement, taking into accounts not only positivity in general, but also HPV types, showed a 65% agreement (complete agreement of 59.7%, partial agreement of 5.3%) and complete disagreement of 35.0%. HPV detection in squamous cell carcinomas (SCC) and adenocarcinomas (ADC) was similar in fresh tissue or paraffin blocks ($p \geq 0.05$).

p16 immunostaining in samples that had at least one HPV negative results showed that 24 out of 25 cases had an over-expressed pattern.

C**onclusions**

HPV DNA detection was lower among ADC as compared to SCC. However, such differences were minimized when additional p16 testing was added, suggesting that the technical issues may largely explain the HPV negative cases.

Background

Improved DNA retrieval methods have made possible the realization of gene analyses and viral genome identification in archival formalin fixed paraffin embedded (FFPE) tissues [1-4]. A number of methods have been developed over the past few years to detect the Human Papillomavirus (HPV), one of them being Short PCR Fragment-10 (SPF_{10}) Line Probe Assay system (LiPA) [5,6]. This assay is based on the amplification of a 65-bp region of L1 open reading frame. In order to validate the SPF_{10} LiPA assay using FFPE cervical samples, comparisons with other samples have been performed by some investigators [7,8] with good overall agreement. The overall agreement was generally high from these studies when cervical scrapes or cytological samples were used. Besides the generally lower detection of HPV in FFPE samples of cervical carcinoma, it has been shown that adenocarcinomas show an even lower positive rate compared to other histological types [9]. In view of these discrepancies, we thought it worthwhile to conduct a study to clarify whether the formalin fixation could differentially affect HPV detection according to the histological diagnosis. The main objective of the present study was to compare HPV detection using SPF_{10} broad-spectrum primers Polymerase Chain Reaction (PCR) followed by Deoxyribonucleic Acid Enzyme Immunoassay (DEIA) and genotyping by $LiPA_{25}$ (version 1) between fresh cervical tissue samples and paraffin embedded blocks from the same patients, and also to assess whether there are differences in HPV detection between squamous cell carcinomas and adenocarcinomas.

Methods

The materials for this study were obtained as part of a case-control study conducted at Mulago Hospital, Kampala, Uganda, which is the teaching hospital for the College of Health Sciences of Makerere University from September 2004 to December 2006. Tumour specimens were obtained from new incident cases of cervical carcinoma presented to the hospital as part of routine care. The tumour specimens were divided into two parts. One part was fixed in 10% formalin and entered in the register of Department of Pathology for processing (paraffin blocks), while the other part was stored at -80°C until transferred to the laboratory for analysis. In cases where the tissue was deemed to be very small, the whole tissue was formalin fixed and processed for histopathological diagnosis. Each case was given an identification number so that the paraffin block could be linked with the fresh specimen. The samples from the cases were all biopsies. The local histopathological diagnosis was performed by Michael Odida.

HPV detection of cervical cancer cases in freshly frozen tissue

HPV detection and genotyping in 195 freshly frozen cervical cancer specimens was performed at the DDL Diagnostic Laboratory in The Netherlands during the period from June to August 2008, also 1,5 up to 4 years after diagnosis. HPV detection was performed using SPF_{10} broad-spectrum primers PCR followed by DEIA and genotyping by $LiPA_{25}$ (version 1).

HPV detection of cervical cancer cases preserved in paraffin

The paraffin embedded blocks were prepared in Uganda for diagnostics purposes between September 2004 and December 2006 when the patients were recruited for the study. HPV detection and genotyping of 201 paraffin blocks was performed by pathologists at the Unit of Infections and Cancer at the Catalan Institute of Oncology (ICO) during January 2010, also 3 to 5.5 years after diagnosis. The detailed methods have been previously described in Odida et al. [10]. Briefly, at ICO the samples were processed following the next steps: (a) Re-embedding of the tissue material was done if necessary when the paraffin block was in poor condition for cutting; (b) Microtome sectioning of the specimens under non-contamination conditions and the sandwich technique were carried out to confirm an optimal number of sections to be used for DNA extraction and testing; (c) All cases were reviewed by a trained pathologist at ICO for diagnosis and assessing quality of the specimen before HPV testing. Cases difficult to classify, cases with a discordant diagnosis compared to the field diagnosis and all the rare histological types were further reviewed by two senior expert pathologists at ICO; (d) DNA was extracted under non-contamination protocols and aliquoted, and HPV testing was performed on each specimen using the SPF-10 broad spectrum primers PCR followed by DEIA. HPV DNA positive samples were subsequently analyzed by $LiPA_{25}$ (version 1: produced at Laboratory Biomedical Products, Rijswijk, The Netherlands), a reverse hybridization technique that detects 25 high-risk (HR) and low-risk (LR) HPV types (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 74). Both labs, DDL and ICO, performed routine cross-validation testing for SPF_{10}, DEIA and LiPA with high agreement between them, concordant results of > 98% (data not shown).

Since the number of samples received at DDL and ICO differ, the comparison was performed based on the cases that were HPV analyzed in both laboratories, n = 171. Data generated at Makerere Hospital (original pathological diagnosis), at DDL (HPV detection in freshly frozen tissue) and at ICO (pathological diagnosis and HPV detection in paraffin blocks) were merged into one dataset using the common identifier first provided by Makerere University. A descriptive analysis of global HPV positivity and HPV types globally and by histological diagnosis of cervical carcinoma and type of specimens was performed. Global HPV detection concordance analysis, in terms of positive or negative result was done to assess differences between the fresh and paraffin, as well as between squamous cell carcinoma (SCC) and adenocarcinomas (ADC).

Further analysis for the overall agreement, taking into account not only general positivity but also HPV types, was done to assess the exact agreement between the two samples. The agreement was classified as complete, partial or complete disagreement according to the definitions below.

Complete agreement: The result is exactly the same (same HPV single type, or same multiple types or HPV negative result).

Partial agreement: The cases have at least one identical type.

Complete disagreement: The cases are totally different (different type or HPV positive-HPV negative).

p16 Immunohistochemistry

It has been largely described that p16 over-expression is a surrogate marker of HPV E7 oncoprotein-mediated catabolism of pRb in premalignant and malignant lesions of the

cervical mucosa. In the present study, p16 immunostaining was performed in a selected number of paraffin embedded tissue from cancer cases in order (1) to obtain more insight regarding the HPV oncogenic role in the discordant cases and (2) to discard non-HPV related cancers in the concordant HPV negative cases. The cases included in this analysis were all HPV discordant cases (n = 24), all concordant HPV negative cases (n = 6) and a random sample of approximately a 10% of the concordant HPV positive cases (n = 15). p16INK4a was detected using the CINtec histology kit (clone E6H4, MTM Laboratories, Heidelberg, Germany), following the manufacturer's protocol. In each series, a negative control and a positive control consisting of an invasive cervical carcinoma were included. The percentage of stained cells (-: < 5%; +: 5-25%; ++: 26-75%; +++: > 75%) and the pattern (focal/diffuse) was recorded, and a positive case was considered when the percentage was 5 and above (++ or more) with a diffuse pattern.

Statistical tests

The Kappa statistics and McNemar test were used to test the statistical significance of HPV detection between fresh samples and paraffin blocks, while the Chi square test and Fisher exact test were used to test the statistical significance of HPV detection between SCC and ADC in both fresh and paraffin blocks. Where appropriate, 95% confidence intervals (CI) were computed, and the level of statistical significant test was set at 0.05.

Results

Overall agreement between fresh tissue specimens and paraffin embedded tissue

Overall, 154 of freshly frozen tissue were positive for HPV (90.1%, 95% CI: 84.6%-94.1%) and 152 of tissue in paraffin blocks were HPV positive (88.9%, 95% CI: 83.2%-93.2%), giving an overall agreement in HPV detection between fresh tissue and paraffin blocks at 86.0% (95% CI: 79.8%-90.8%), which was statistically significant (Kappa Index = 0.26, p = 0.001). Although the proportion of HPV positive cases in fresh tissue was higher than those in paraffin blocks, the difference was not statistically significant (McNemar test, $p > 0.05$). The details are shown in Table 1.

In both types of tissues, single HPV infections were predominant. Only one case has been DEIA positive, and LiPA negative (HPVX, unknown type) and has been detected in paraffin embedded tissue. The proportion of multiple HPV infections was higher in freshly frozen tissue cases than tissue in paraffin blocks. However, the differences between both kinds of tissues were not statically significant (all p-values are > 0.05). The details are shown in Table 2.

HPV types in single infections

In both kinds of tissue, HPV16 was the most frequently identified in 47.4% among HPV positive cases. The proportion of HPV33, HPV52 and HPV58 were almost identical. A higher proportion of HPV18 and HPV35 were identified in paraffin blocks compared to fresh tissue and, conversely, a higher proportion of HPV45, HPV68 or 73, HPV51 and

HPV39 in fresh tissue. HPVs 66 and 31 were only identified in fresh tissue and HPVs 59, 11 and HPVX were only detected in paraffin embedded tissue. The details are shown in Figure 1.

HPV types in multiple infections

Table 3 shows HPV type distribution of multiple types in freshly frozen tissue versus tissue in paraffin blocks. In 11 out of the 16 cases described in table 4, the HPV result matched at least for one of the HPV types.

HPV types agreement between fresh tissue specimens and paraffin embedded specimens

When analyzed for the overall agreement, also taking into account HPV types, complete agreement was found to be 59.7% (complete agreement in single HPV types: 55.0%, complete agreement in multiple types: 1.2%, complete agreement in HPV negativity: 3.5%), partial agreement: 5.3% and complete disagreement: 35.0%.

HPV detection concordance by histological type of cervical carcinoma

For performing the HPV detection concordance analysis stratified by histological diagnosis, we selected the cases that had a concordant histological diagnosis between the local and ICO pathology evaluation: SCC (n = 130) and ADC (n = 7).

Sqamous cell carcinoma

The HPV positivity in freshly frozen tissue was 91.5% (95% CI: 85.4%-95.7%), and the corresponding HPV positivity in paraffin embedded tissue was 93.1% (95% CI: 87.3%-96.8%), giving an overall agreement in HPV detection between fresh tissue and paraffin embedded tissue of 90.8% (95% CI: 84.4%-95.1%), which was statistically significant, although the strength of concordance is weak (Kappa Index of agreement of 0.35; $p < 0.001$). Although identifying a higher HPV positivity in tissue from paraffin blocks, this difference was not statistically significant (McNemar test, $p > 0.05$).

Adenocarcinoma

The HPV positivity in freshly frozen tissue was 85.7% (95% CI: 42.1%-99.6%), and the HPV positivity in paraffin embedded tissue was 71.4% (95% CI: 29.0%-96.3%), giving an overall agreement in HPV detection between fresh tissue and paraffin blocks: 85.7% (95% CI: 42.1%-99.6%), which was not statistically significant (Kappa Index of agreement of 0.59; $p > 0.05$). Although identifying a higher HPV positivity in fresh tissue, this difference was not statistically significant (McNemar test, $p > 0.05$).

HPV detection concordance between histological types of cervical carcinoma

Comparing HPV positivity by diagnosis and tissue preservation, adenocarcinomas were more likely to be HPV negative than SCC, both in freshly frozen tissue and paraffin embedded tissue. This difference was higher in paraffin embedded tissue than in fresh tissue (21 percentage points difference versus 6, respectively), although the differences were not statistically significant ($p > 0.05$), as is shown in Table 4.

p16 Immunostaining results

45 cases were included in the p16 analysis. From these cases, p16 was successfully performed in 40 (88.9%). In the other 5 cases, there was no tumour in the p16 slide (11.1%). All cases except one showed a positive p16 over-expressed pattern (Table 5). Only one case, an adenocarcinoma NOS (subtype: not other specified), was HPV negative in both fresh and paraffin embedded tissue and also showed a p16 negative pattern, suggesting that maybe it is not HPV related or it is a case with an endometrial origin (not cervical).

Discussion

The goal of this study was to examine whether HPV genotyping on FFPE invasive cervical carcinoma specimens give comparable results with freshly frozen specimens obtained from the same patient simultaneously. Our study suggests that detection of HPV using SPF_{10} with LiPA technology using formalin fixed paraffin embedded tissues gives comparable results to freshly frozen specimens for HPV detection in general (Kappa index 0.26, p = 0.001), see Table 1, but is not so good for specific HPV types detection (p-value > 0.05). Some previous studies which compared the paraffin embedded tissue with other samples found high agreement [7,8,11]. However, these studies used exfoliated cells or cervical scrapes and not freshly frozen specimens. Besides, these were cases of preneoplastic lesions, not invasive cervical carcinoma. A study which compared FFPE tissue with cervical scrapes from women with invasive cervical carcinoma found very high HPV type specific concordance [12]. However, the number of samples studied was small, and also the method of detection was GP5+/6+ primers.

Another interesting observation was the negative HPV results in both freshly frozen specimens and FFPE tissues. Our analysis of p16 immunostaining in samples with at least one negative result showed that all but one sample tested p16 positive, suggesting that the cases are HPV mediated and that the most probable reason for not detecting the virus is related to viral, specimen preservation or technical issues such as denatured DNA by alkali [13] or by formalin fixation [14]. For specific HPV types, if one considers fresh specimens as gold standard, then discordance in HPV results from FFPE specimens could be due to a number of explanations. One is that formalin fixation could have denatured

the tissue. It is known that DNA extracted from FFPE tissues are usually at low concentration and fragmented [14,15]. Another alternative view is reduced sensitivity detection of some HPV types in paraffin specimens, especially types 42, 16, 18, 39, 56, 58, 59 and 66 [16-18]. A third possibility, which may be applicable to discordance in multiple infections is due to competition between different HPV genotypes [19]. It is conceivable that lower discordance rates could be obtained by reducing the duration of fixation in buffered formalin.

The low detection of HPV in adenocarcinoma in both types of specimens is also of interest. A number of previous studies have also found low detection of HPV in adenocarcinoma of the cervix [9,20,21]. Plausible explanations are: (a) that some cervical tumours may be of endometrial origin and are therefore HPV negative [22]; (b) that some cervical adenocarcinomas may not be related to HPV (such as minimal deviation adenocarcinomas) [21,23]; (c) low viral load; (d) fewer episomal copies; (e) or loss of viral genome during integration in cervical adenocarcinoma [24]. Though some investigators found a high positive rate which they attributed to higher diagnostic accuracy and exclusion of non cervical tumours [25].

Our results have some implications. For areas with low resources, use of FFPE samples for HPV detection is feasible, especially HPV testing for clinical management of women with abnormal smears. In case a biopsy is taken, the paraffin embedded tissue could be referred to laboratory for HPV testing. This would make use of HPV results, whether for prognosis or detection of progression of cervical lesions like high-grade squamous intraepithelial lesion (HSIL) possible in many areas. However, there would be a need to have standard protocols so that fixation time is controlled. In some situations, the

preference for fresh or paraffin tissue will depend on the amount of available tissue and the questions being addressed.

Our study had some strengths. Compared with a recent study which compared the use of cervical scrapes and tissues from women with cervical carcinoma [12], our sample size was big. The specimens were analyzed in two different laboratories blindly using the same HPV detection method. The SPF_{10} and LiPA technology are quite sensitive and specific for HPV [16]. The study also has, however, some limitations. The piece of tissue used was divided in two, and half was used to prepare the paraffin blocks and half kept as freshly frozen tissue for HPV genotyping. Thus, they were not exactly the same tumour materials, but pieces of tissue adjacent to each other. Moreover, a small number of adenocarcinomas limits the use of statistical tests.

Conclusions

There were no differences in HPV positivity between freshly frozen tissue and paraffin embedded tissue in general, but for specific types, only 65% of the cases had complete or partial agreement on the HPV detection. HPV DNA detection was lower in ADC as compared to SCC, the difference being higher in paraffin blocks than in fresh tissue (21 percentage points difference versus 6, respectively; $p > 0.05$). However, such differences were minimized when additional p16 testing was added, suggesting that technical issues largely explain the HPV negative cases.

List of abbreviations

ADC, adenocarcinoma; CI, confidence interval; DDL, Diagnostic Laboratory; DEIA, deoxyribonucleic acid enzyme immunoassay; FFPE, formalin fixed paraffin embedded; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; ICO, Catalan Institute of Oncology; INK4, tumour suppressor protein; LiPA, line probe assay; p16INK4a, prototypic INK4 protein on p16 gen, identified as a tumour suppressor in many human cancers; PCR, polymerase chain reaction; SCC, squamous call carcinoma; SPF_{10}, short PCR fragment;

Competing interest

The authors declare no competing interests.

Authors' contributions

MO, EW and SdS were responsible for developing concept, full proposal development and getting ethical approvals. MO was responsible for samples selection, histological review of origin and data cleaning. SS, BQ and LA were responsible for data analysis. BL and MA were responsible for histological review of the paraffin cases and p16 analysis. WQ, BK and LJvD were responsible for HPV DNA detection in fresh samples. MO, EW and SdS wrote the manuscript. All co-authors: SS, BL, BQ, LA, WQ, BK, LJvD, MA revised and gave valuable comments to the first draft of this manuscript. MO and EW were responsible for preparing the manuscript for submission. All authors approved the final version submitted.

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Figure legends

Figure 1 - HPV type distribution of single types in fresh tissue versus paraffin block

Percentiles (%) of single/multiple/X HPV types among HPV positive cases

Tables

Table 1 - HPV detection in fresh tissue versus paraffin block

		Paraffin block				Total	
		Positive		Negative			
		N	% [a]	N	% [a]	N	% [a]
Fresh tissue	Positive	141	82.5[b]	13	7.6	154	90.1
	Negative	11	6.4	6	3.5[b]	17	9.9
	Total	152	88.9	19	11.1	171	100

[a] Percentage of cell number among all the cases (171)

[b] Concordant results

Concordance analysis-Kappa Index: value of the index 0.26; p = 0.001

McNemar test: p > 0.05

Table 2 - HPV detection in fresh tissue versus paraffin block: single/multiple/HPVX types

HPV detection	Fresh tissue			Paraffin block		
	Number of cases	%	95% CI[b]	Number of cases	%	95% CI[b]
Cases HPV analyzed	171	100		171	100	
HPV negative	17	9.9	(5.9-15.4)	19	11.1	(6.8-16.8)
HPV positive	154	90.1	(84.6-94.1)	152	88.9	(83.2-93.2)
Single HPV types[a]	143	92.9	(87.6-96.4)	144	94.7	(89.9-97.7)
Multiple HPV types[a]	11	7.1	(3.6-12.4)	7	4.6	(1.9-9.3)
HPV X – unknown[a]	0	0.0	(0.0-2.4)	1	0.7	(0.0-3.6)

CI, Confidence Interval;

[a] Number of single/multiple/X among HPV positive cases;

[b] For all comparisons: p-value > 0.05

Table 3 - HPV type distribution of multiple types in fresh tissue versus paraffin block

Fresh tissue[a]	Paraffin block[b]
HPV **18 & 74**	HPV **18 & 74**
HPV **16 & 52**	HPV **16 & 52**
HPV **16**	HPV **16** & 35
HPV **18**	HPV 16 & **18**
HPV **18**	HPV **18** & 56
HPV **45**	HPV **45** & 54
HPV **16** & 45	HPV **16**
HPV **18** & 31	HPV **18**
HPV **18** & 70	HPV **18**
HPV **59** & 74	HPV **59**
HPV **39**	HPV 16 & **39**
HPV 16 & 18	HPV 59
HPV 18 & 52	HPV 16
HPV 6 & 44 & 45	HPV 18
HPV 39 & 51 & 52	HPV 33
HPV 6 & 45	HPV negative

[a] Multiple HPV cases, N = 11

[b] Multiple HPV cases, N = 7

Table 4 - HPV detection in fresh tissue versus paraffin block according to histological diagnosis

HPV detection	SCC N = 130			ADC N = 7		
	Number of cases	%	95% CI	Number of cases	%	95% CI
Fresh tissue	119	91.5	(85.4-95.7)	6	85.7	(42.1-99.6)
Paraffin blocks	121	93.1	(87.3-96.8)	5	71.4	(29.0-96.3)

ADC, adenocarcinoma; CI, confidence interval; SCC, squamous cell carcinoma;

For all comparisons: p-value > 0.05

Table 5 - Results of p16 expression by HPV results

HPV results fresh/paraffin preservation	p16 -		++		+++		Total	
	N	%	N	%	N	%	N	%
Concordant HPV negative	1[a]	16.7	1[b]	16.7	4[e]	66.7	6	100
Concordant HPV positive	0	0.0	1[c]	6.7	14[f]	93.3	15	100
Fresh HPV positive, Paraffin HPV negative	0	0.0	0	0.0	9[g]	100	9	100
Fresh HPV negative, Paraffin HPV positive	0	0.0	2[d]	20	8[h]	80	10	100
Total	1	2.5	4	10	35	87.5	40	100

p16-: < 5% stained cells; p16++: 26-75%; p16+++: > 75%

Histologies:

[a] adenocarcinoma NOS (not other specified)

[b] undifferentiated carcinoma

[c] undifferentiated carcinoma

[d] 1 adenocaricnoma and 1 undifferentiated carcinoma

[e] 4 squamous cell carcinoma

[f] 13 squamous cell carcinoma and 1 undifferentiated carcinoma

[g] 5 squamous cell carcinoma, 1 adenocarcinoma and 3 undifferentiated carcinoma

[h] 7 squamous cell carcinoma, 1 adenocarcinoma

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