ABSTRACT

Background

Human papilloma virus (HPV) has been associated to conjunctival squamous cell carcinoma (CSCC) and has been documented in studies originating from different parts of the world. In Zambia, eye cancers are on the increase and between 2005 and 2009 they were ranked number three (3) among the top five (5) cancers of which conjunctival squamous cell carcinoma (CSCC) (78%) was the commonest. In this study, the aim was to establish the prevalence and association of HPV infection with CSCC at the University Teaching Hospital (UTH) and Lusaka Adventist Eye Hospital, Zambia.

Materials and Methods

This was a cross section study conducted at UTH and Lusaka Adventist Eye Hospital, Zambia for a period of three (3) months. A convenient sampling method was used. Conjunctival biopsies were collected from patients aged over 20 years with clinically suspected CSCC and met an inclusion criteria making the sample of 37 participants. Histopathology and polymerase chain reaction assay (PCR) analysis of 37 specimens of conjunctival lesions was undertaken. Tissues confirmed as CSCC (21) were grouped as cases and non-CSCC tissues (16) as controls. Findings were analysed using statistical software SPSS for windows version 18.0. Univariate analysis of variables which were CSCC, HPV, and HIV was conducted to determine the distribution patterns of HPV and HIV. Odds ratios (ORs) and 95% confidence intervals (CIs) were computed stratifying by age, sex, human papilloma virus and HIV status.

Results

This study revealed that, 59.5% of the conjunctival tissues were malignant of which 95.5% were CSCC and 4.5% were Kaposi's sarcoma. Most of the participants 57% were HIV positive. 71.4% of patients with CSCC were HIV positive and HPV DNA was detected in 71.4% of CSCC tissues. HPV DNA was detected in 52.4% HIV+ patients and in 62.5% HIV- patients.

Conclusion

The study showed a high prevalence of HPV infection (71.4%) in CSCC. HPV and HIV infections were both associated with conjunctival SCC. Though HPV infection was found in patients with HIV, there were no significant differences in the results between HIV positive and HIV negative patients.

Keywords: conjunctival squamous cell carcinoma, human papilloma virus, polymerase chain reaction.

DEDICATIONS

This dissertation is dedicated to my late wife, Mrs. Elizabeth Katongola Chapima who could not live to see my graduation and all people affected with cancers.

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TABLE OF CONTENTS

CONTENT **PAGE NO**

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
CIN	Conjunctival Intraepithelial Neoplasia
CSCC	Conjunctival Squamous Cell Carcinoma
H&E	Haematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma virus
ISH	In-Situ Hybridisation
OR	Odds Ratio
OSSN	Ocular Surface Squamous Neoplasia
PCR	Polymerase Chain Reaction
SCC	Squamous Cell Carcinoma
UNZA	University of Zambia
UTH	University Teaching Hospital
UV	Ultraviolet
ZNCRO	Zambia National Cancer Registry Office

CHAPTER 1.

1.1 Background

Conjunctival squamous cell carcinoma (CSCC) is one of the malignant tumours that grow on the outer surface of the eye. CSCC is described in the ophthalmic literature as a rare and slow-growing tumour caused by chronic exposure to ultraviolet (UV) light affecting elderly men of above 70 years of age (Lee and Hirst, 1995).

It has been observed that, since the 1980s, the number of patients presenting with CSCC has been increasing rapidly worldwide (Poole, 1999). The cause of this increase is not known and this has led to the generation of several hypotheses which includes the association of CSCC to infective agents such as human papilloma virus (HPV) infection (especially type 16 and 18) and the human immunodeficiency virus (HIV) (Deniz, 2010).

HPV is a DNA virus from the Papovaviridae family that has been identified in a variety of epithelial lesions of the skin and mucosa such as the cervix, anogenital, oesophagus and conjunctiva. There are over 100 HPV types which have been identified so far, and the epidemiological classification currently includes three groups namely: The high-risk types which are types; 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, 73 and 82; probable risk types which are types; 26, 53 and 66 and the low-risk types which are types; 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 (Lungu, et al, 1992). It is common for an epithelium to be infected by more than one HPV type, particularly in low-grade lesions.

Some of the HPV cause malignant transformation in a tissue by binding tumour suppressor proteins in the host, therefore affecting the cellular cycle of the infected cells (Burd, 2003). HPV's E6 and E7 proteins interact with key cell cycle proteins

including pRB and p53, effectively over-riding the G1/S-phase checkpoint. Binding of E7 to pRB activates the E2F transcription factor, which triggers the expression of proteins necessary for DNA replication (Munger et al, 2004).

Unscheduled S-phase would normally lead to apoptosis by the action of p53; however, in HPV-infected cells, this process is counteracted by the viral E6 protein, which targets p53 for proteolytic degradation (Thomas et al, 1999). As a result, the dependence on cell cycle control is abolished and normal keratinocyte differentiation is retarded (Doorbar, 2005). A continual activity of the viral proteins E6 and E7 leads to cumulative genomic instability, accumulation of oncogene mutations, further loss of cell-growth control, and finally cancer.

HIV is the virus that leads to acquired immune deficiency syndrome (AIDS) due to immunosuppression. The potential for HIV to cause cancer is due to the reduction in the effectiveness of the immune surveillance system resulting in the unchecked growth of the tumour. (Smith, et al, (2010).

It has also been reported that, many people infected with HIV are also infected with other viruses that cause certain cancers (Engels et al, 2008). HPV is one of such viruses (Luchters, et al. 2010 and Smith, et al. 2010). Hypothetically, cervical HPV infection predispose uninfected woman to HIV by recruiting increased number of HIV target cells such as T lymphocytes to the genital mucosa. Although HPV does not produce ulcerative genital lesions or exudates, recruitment of these cells to the genital epithelium is required to clear the infection. Kobayashi et al, 2004, found that HPV-associated dysplastic cervical lesions were associated with an increased number of CD4 + T cells when compared to normal cervical epithelium. This is because HPV, like most other intracellular viral infections, requires activated T-cells to elicit an effective cell-mediated immune response (Averbach et al, 2010). Therefore, it is biologically reasonable that HPV infection enhances a woman's susceptibility to HIV acquisition by exposing target cells to HIV in male semen.

This study, set out to establish the prevalence and association of HPV in CSCC and further determined whether HPV infection co-existed with HIV infection.

1.2 Statement of the Problem

According to the Zambia National Cancer Registry Office (ZNCRO) (from 2005 to 2009), eye cancers were ranked number 3 (11%) among the top five (5) cancers in Zambia as indicated in the table below.

Table 1.1: Top five (5) Cancers in Zambia between 2005 and 2009

TYPE OF CANCER	2005	2006	2007	2008	2009	TOTAL
Cervix	388 (45%)	581 (41%)	745 (58%)	763 (62%)	770 (62%)	3247 (54%)
Kaposis Sarcoma	248 (29%)	441 (32%)	136 (11%)	115 (9%)	144 (12%)	1084 (18%)
Eye	119 (14%)	159 (11%)	164 (13%)	106 (9%)	91 (7%)	639 (11%)
Breast	36 (4%)	124 (9%)	131 (10%)	141 (12%)	130 (10%)	562 (9%)
Prostate	67 (8%)	102 (7%)	97 (8%)	102 (8%)	108 (9%)	476 (8%)
TOTAL	858(100%)	1407(100%)	1273(100%)	1227(100%)	1243(100%)	6008 (100%)

Source: Zambia National Cancer Registry, 2012

It was also noted that, eye cancers were slightly more common in men than women except in 2009 when more women had eye cancers as shown in table 2 below.

Table 1.2: Prevalence of eye cancers according to sex (2007 – 2009)

YEAR	MALE	FEMALE	TOTAL
2007	92 (56%)	72 (44%)	164 (100%)
2008	64 (60%)	42 (40%)	106 (100%)
2009	44 (48%)	47 (52%)	91 (100%)
TOTAL	200 (55%)	161 (45%)	361 (100%)

Source: Zambia National Cancer Registry, 2012

Statistics obtained from the University Teaching Hospital (UTH) pathology laboratory showed a high number of CSCC cases in the past 3 years as indicated in table 3.

Table 1.3: Confirmed CSCC at UTH (2008 – 2010)

YEAR	CSCC
2008	78
2009	73
2010	64
TOTAL	**215**

Source: UTH Pathology Laboratory Records, 2012

When data from ZNCRO and UTH pathology laboratory were analysed, it was observe that, CSCC was the most common eye cancer compared to other eye cancers in Zambia as indicated in table 4 below.

Table 1.4: Prevalence of confirmed CSCC between 2008 and 2009 at UTH Compared to other Eye Cancers in Zambia

YEAR	CSCC	*NON-CSCC	TOTAL
2008	78 (74%)	28 (26%)	106 (100%)
2009	73 (84%)	14 (16%)	91 (100%)
TOTAL	151 (78%)	42 (22%)	197 (100%)

**** Retinoblastoma, lacrimal sac lesions, ocular Kaposi's sarcoma etc.***

From the Statistics, it is clear that, CSCC (78%) is a major public health problem in Zambia among all eye cancers. The effects of CSCC on Zambian people can have both economic and health implications. Economic effects include costs related to illness such as treatment, care and transport to and from the health facility. Health burdens may include the impact of cancer on health-related quality of life as measured in terms of disability-adjusted life years and quality-adjusted life years on the individual patient.

1.3 Justification and Significance for the Study

The association of HPV with CSCC and co-infection of HPV with HIV has been documented in various studies originating from different parts of the world. However, no such studies have been done in Zambia. This may have created a gap between knowledge and evidence based medical practice in Zambia. Since the effective control of CSCC depends on the identification of the most common causes and risk factors, it was important to undertake this study to find out if there is an association between HPV and CSCC in Zambian population. It was also important to establish whether there was an HPV co-infection with HIV in patients with CSCC. The information from this study will be used to fill in the gaps in current knowledge and practices in the evaluation and formulation of treatment and preventive strategies of conjunctival squamous cell carcinoma.

CHAPTER 2. LITERATURE REVIEW

2.1 Prevalence and association of HPV with CSCC

HPV infection is believed to be one of an important factors in the development of cancer of the conjunctiva and it has been detected in 44 – 92% of CSCC using various detecting methods (Lass, et al., 1995).

In a study conducted by McDonnell, et al. (1987) in Baltimore, using in-situ hybridization method, it was reported that 53% (15/ 23) of conjunctival papillomas harboured HPV DNA. Type 6 was the common HPV DNA detected. In this study, it was concluded that infection with HPV 6 was responsible for most of the conjunctival papillomas.

The same method was used by Moubayed, et al. (2004), in Tanzania, in which 14 tissues of conjunctival epithelial dysplasia and epithelial neoplasms were examined for the presence of HPV DNA. The report indicated that all of them (100%) tested positive of HPV DNA. HPV type 16 and 18 were the common HPV DNA detected. In each case, histological evaluation revealed a transitional lesion extending from koilocytic dysplasia to severe dysplasia or invasive squamous cell carcinoma. These observations seemed to suggest the central role of HPV 16 and 18 in the oncogenesis of conjunctival cancers in the subtropical Tanzania.

Saegusa, et al. (1995) conducted a study in Japan to clarify the role of HPV infection in the development of papilloma, dysplasia, squamous cell carcinoma, and basal cell epithelioma arising from the eyelids and the conjunctiva. Sixteen cases of papilloma, 4 of dysplasia, 4 of squamous cell carcinoma, and 12 of basal cell epithelioma were examined using formalin fixed and paraffin embedded samples. Detection of HPV-DNA was performed by PCR-RFLP and in-situ hybridisation (ISH) methods. HPV 16

was detected in 12/16 (75%) papillomas, 2/4 (50%) dysplasias, and 1/4 (25%) squamous cell carcinomas but none in basal cell epitheliomas. No other HPV subtypes were found in this study. ISH assay showed positive signals in only two cases of dysplasia and squamous cell carcinoma. Based on the presence of HPV 16 in both benign and malignant lesions in this study, it was concluded that HPV 16 alone may be incapable of causing conjunctival squamous cell carcinoma, and that any correlation between the papilloma-squamous cell carcinoma sequence and HPV infection may have been due to chance.

In a study conducted by Nakamura, et al. (1997) in Japan to determine the relation between CSCC and HPV infection, 17 individuals with such tumours were investigated. Nine of the seventeen tumours were benign, four were dysplastic lesions, and four were carcinomas. In this study eight specimens showed positive immunohistochemical staining with antibodies to HPV; four of these eight were papillomas, three were dysplastic lesions, and only one was a carcinoma. Koilocytosis was detected in seven of these eight tumours. Five of the eight specimens positive for immunohistochemical staining were also positive for HPV DNA by ISH, and all eight were positive for HPV DNA by PCR method. This study indicated that at least 50% of squamous tumours of the ocular surface and lacrimal sac lesions were associated with HPV infection. The study also showed that PCR was highly sensitive for detection of HPV DNA.

Tabriz, et al. (1997) in Australia also conducted a study to investigate the role of human HPV types, 6, 11, 16 and 18 in corneal and conjunctival carcinomas. In this study, 88 dysplastic corneal and conjunctival specimens and 66 controls that had been formalin-fixed and paraffin-embedded were examined. Sections were graded for histological

abnormality by light microscopy and the presence of HPV DNA was determined by PCR. HPV DNA was detected in 34 (39%) dysplasias and in 5 (7.5%) controls. Out of the 34 dysplasias that were HPV positive, 20 (59%) contained either types 16 or 18, 13 (38%) contained types 6 or 11, while combinations of HPV types were present in 11 (32%). The results indicated that 39% (34 out of 88) corneal and conjunctival carcinomas harboured HPV DNA. However, this study reported an unexpectedly high incidence of HPV 6 and 11 in conjunctival carcinomas, which may suggest that they too have a role in the development of corneal and conjunctival carcinomas.

Karcioglu and Issa (1997) in Saudi Arabia conducted a study to detect HPV types 16 and 18 in neoplastic and non-neoplastic disorders of the external eye. The study examined 96 paraffin embedded tissue specimens with neoplastic and non-neoplastic lesions and 19 conjunctiva samples free from overt disease obtained from patients during routine cataract extractions. HPV types 16 and 18 DNA were identified in 57% of in situ squamous cell carcinoma, 55% of invasive squamous cell carcinoma and in 32% of normal conjunctival tissues. These findings indicate that HPV types 16 and 18 are detectable with the polymerase chain reaction not only in epithelial neoplasms of the ocular mucous membrane but also in non-neoplastic lesions as well as in apparently healthy conjunctiva. This study concluded that, HPV may be involved in the development of CSCC.

In Brazil, Palazzi, et al. (2000) conducted a study to evaluate the presence of HPV DNA in acquired lesions of the conjunctiva and in normal mucosa. Thirty patients with acquired lesions of the conjunctiva and sixty matched controls (by age and sex) were evaluated. HPV DNA in each of the normal conjunctiva and in acquired lesions was detected by the PCR technique and dot blot hybridization. The material was collected

by scraping the normal mucosa and the surface of the lesions. A fragment of fresh frozen tissue and paraffin embedded specimens of each lesion were also included. The results indicated that HPV DNA was detected in four of the 31 lesions evaluated (12.9%) and in the healthy mucosa of one individual of the control group (1.6%). HPV type 16 was detected in two carcinomas and in the normal mucosa of one individual of the control group. HPV type 11 was demonstrated in two papillomas of one patient with lesions in both eyes. The low frequency of HPV DNA found in the lesions of this sample and the detection of the viral genome in the normal mucosa in this study indicates that there could be an association between HPV infection and the carcinoma of the conjunctiva in Brazil.

In a study conducted by Sjo, et al. (2001) 55 archival paraffin embedded tissue from conjunctival papillomas were analysed for the presence of HPV by PCR and subsequent filter hybridisation. This study was conducted in Denmark. Histological sections of the 55 papillomas were evaluated for the presence of koilocytosis. The study showed that HPV was present in 48 of 52 (92%) conjunctival papillomas. HPV type 6 and 11 were found in 40 of investigated papillomas and a double infection with HPV 6 and 16 was identified in a single papilloma. In six papillomas, the HPV type was not identified. Koilocytosis was present in 22 of 55 papillomas (40%). These results indicated a strong association between HPV and conjunctival papillomas. HPV type 6 and 11 (low risk group) was the most common HPV which was detected. However, the sensitivity of koilocytosis as an indicator of HPV in conjunctival papilloma was low.

In another study conducted by Sjo, et al. (2007) in Denmark, 165 conjunctival papillomas and 20 histologically normal conjunctival biopsy specimens were analysed

for the presence of HPV using PCR. HPV DNA was present in 81% (86 of 106) of papillomas. HPV type 6 was positive in 80 cases, type 11 in 5 cases and type 45 was present in a single papilloma. All the 20 normal conjunctival biopsy specimens were HPV negative. This study agreed with the earlier study, which indicated a strong association between HPV and conjunctival papillomas. This study also correlated with the study that was done by Scott, et al., (2007) in USA.

In a study to identify the roles of HPV infection and solar elastosis as risk factors for CSCC by Tulvatana, et al. (2003), 60 tissues were examined. Thirty were consecutive pathological specimens, ranging from conjunctival intraepithelial neoplasia, carcinoma in situ, to invasive squamous cell carcinomas, which were retrieved from tissue archives. Thirty controls were disease free conjunctiva from age and sex matched patients undergoing extra capsular cataract extraction. Two masked pathologists studied haematoxylin and eosin stains on paraffin embedded conjunctival tissues. Elastic stain for solar elastosis was blindly interpreted in comparison with negative and positive controls. HPV infection was studied by PCR and dot hybridisation. After examination, solar elastosis, a disorder in which the skin appears yellow and thickened as a result of sun damage, was seen in 53.3% of CSCC and in 3.3% of controls. HPV DNA was not detected in any of the specimens. In this study, solar elastosis was much more frequently found in CSCC cases than in their matched controls and was a risk factor for CSCC. This study concluded that chronic exposure to sun light may be associated with CSCC.

In India, Sen, et al. (2007) conducted a retrospective study to determine the association of HPV in human conjunctival neoplasms. Histopathological and immunohistochemical analysis of 65 specimens of ocular neoplasia and 30 normal

controls diagnosed between 1991 and 2002 at a tertiary eye care hospital was undertaken. Formalin-fixed, paraffin-embedded tissues were reviewed for confirming histopathological diagnosis, presence of koilocytosis and changes related to actinic keratosis. Immunohistochemical analysis was done using HPV-specific monoclonal antibodies. Clinicopathological correlation and the association of HPV antigen with the histopathological features were performed. Out of the 65 cases analysed, 35 were papillomas and 30 were CSCC. Histologically, koilocytosis was observed in 17.1% of papillomas and 36.6% of CSCC. Actinic keratosis was present in 33% of CSCC. Immunohistochemically 17.1% conjunctival papillomas stained positive for HPV antigen, all cases of CSCC were negative for HPV. In this study, there was no correlation between koilocytosis or actinic keratosis and the detection of HPV antigen. According to this study, the association between HPV and conjunctival neoplasms is variable in different geographical areas and depends on the methods of detection used. This study warrants the need to apply more advanced techniques at molecular level to determine the possible aetiology of HPV in conjunctival neoplasms among Asian-Indians.

Amoli, et al. (2011) also conducted a case control study to detect HPV in CSCC in Iran. In this study, 50 CSCC cases (mean age: 65.2) and 50 normal conjunctival biopsies (mean age: 63.8) obtained from the cancer registry archive at Pathology Department of Farabi Eye Hospital were examined for the presence of HPV DNA. MY/GP nested PCR was performed for HPV detection and E6/E7 consensus primers in combination of type specific primers were used in another nested PCR series for HPV typing. HPV DNA was detected in 46/50 (92%) samples of squamous cell carcinoma and not in any of the normal biopsies by nested PCR using primer sets of the HPV consensus L1 region (MY/GP). Subsequently, specimens from the 46 positive

cases were subjected to specific PCR. Although 630bp amplicon was produced in 44 of 46 samples (E6/E7 primers), none of the specific HPV PCR reactions for HPV DNA type 16, 18, 31 or 33 resulted in the detection of HPV DNA in the 44 CSCC specimens. These current results seemed to confirm the role of HPV in the aetiology of conjunctival CSCC. However, the absence of HPV 16, 18, 31 and 33 in conjunctival CSCC in this study raises doubts about the role of genital types of HPV in conjunctival carcinomas which can be due to self-inoculation.

2.2 Co-existence of HPV with HIV infections

HPV and HIV are each considered responsible for a considerable burden of disease and the interactions between these infections pose substantial public health challenges.

Luchters, et al. (2010) conducted a study of 820 female sex workers enrolled between July 2005 and January 2006 to establish the association of HIV infection with HPV infection in Kenya. After interview and a gynaecological examination, blood for HIV and cervical cytology samples were taken for PCR and cytology. Prevalence of high-risk HPV was compared between HIV-infected and uninfected women, and in women with abnormal cervical cytology. 35.2% of women (283/803) were HIV infected. Of the 283 HIV-infected women, 200/283 (73.3%) had high-risk HPV and 35.5% (97/273) had HPV 16 and/or 18. In the HIV-negative women, 229/503 (45.5%) had high-risk HPV and 15.7% (79/503) had HPV 16 and/or 18. The study showed that HIV infected sex workers had almost four-fold higher prevalence of high-risk HPV and more precancerous lesions.

In another prospective study conducted by Smith, et al. (2010), 2040 HIV-negative Zimbabwean women (average age 27 years, range 18–49 years) were tested quarterly for 29 HPV types (with L1 PCR primers) and HIV (antibody testing on blood samples

with DNA or RNA PCR confirmation). HIV incidence was 2.7 per 100 woman-years. The HPV prevalence was 24.5%, and the most prevalent HPV types were 58 (5.0%), 16 (4.7%), 70 (2.4%), and 18 (2.3%). This study showed that cervico-vaginal HPV infection was associated with an increased risk of HIV acquisition in women, and specific HPV type 58 was implicated in this association.

2.3 Conclusion

From the literature review there is overwhelming evidence that, HPV is associated with proliferative ocular surface lesions and can be detected using several methods such as PCR, in-situ hybridization technique and immunohistochemical staining. However, the use of PCR has shown good results and is more sensitive. This study used PCR method because PCR is highly sensitive compared to other methods.

Although HPV had been detected successfully in conjunctival lesions in various studies, these studies faced two limitations. The first limitation was the use of archival material fixed in formalin to evaluate the presence of human papillomavirus DNA using PCR assays. Formalin is a known PCR inhibitor, as it degrades the protein in the tissues (Agaba, 2010). This could have led to inaccurate results. The second limitation was the lack of broad-spectrum highly sensitive PCR tests. These studies used specific primers, which had no ability to test a wide variety of HPV. This also hampered the efforts of researchers to test for a wide variety of HPV. Although HPV is considered as one of the probable etiologic agents in proliferative ocular surface lesions worldwide, no related published data is available from Zambia participants.

2.4 Research Question

Do conjunctival lesions clinically suspected of SCC harbour human papillomavirus DNA in indigenous black Zambian patients?

2.5 General Objective

To establish the prevalence and association of HPV infection with CSCC at UTH and Lusaka Adventist Eye Hospital, Zambia.

2.6 Specific Objectives

1. To examine the presence of HPV DNA in conjunctival lesions clinically suspected of SCC.
2. To determine an association of HPV with CSCC.
3. To determine whether HPV co-exist with HIV in patients diagnosed with CSCC at UTH and Lusaka Eye Hospital.

CHAPTER 3. MATERIALS AND METHODS

3.1 Research Design

A cross-sectional study with quantitative dimensions was used in this study.

3.2 Research Setting

The study was conducted at Lusaka Adventist Eye Hospital and the University Teaching Hospital (UTH), Lusaka, Zambia. These sites were purposefully selected owing to the fact that, UTH is the national referral centre for all cancers and Lusaka Eye hospital being a private hospital sees quite a good number of these cases. It was also selected because of the availability of the facilities and equipments needed to conduct this study.

3.3 Target and Study Populations

The study population was all those that met the eligibility criteria.

3.4 Inclusion criteria

All tissues of patients aged 20 years and above with a known HIV status who presented with clinically suspected CSCC at UTH eye clinic and Lusaka Adventist Eye hospital between May and July 2013 were recruited in the study. Tissues with histologically confirmed CSCC (21) were grouped as cases and non-CSCC as controls.

3.5 Exclusion criteria

Tissues of patients who were below 20 years, those with an unknown HIV status and those with conjunctival lesions but not suspected to be CSCC (pterygium and pingueculum) were not included in the study. All patients who presented with recurrent CSCC and those who had been on radiotherapy or chemotherapy were also excluded

from the study since the initial treatments could can have an effect on the PCR results (Agaba et al., 2010).

3.6 Sample Selection

Convenience sampling was used because the numbers of CSCC cases were limited.

3.7 Sample Size

The sample size consisted of 37 clinically suspected CSCC lesions taken from patients attending eye clinic at UTH and Lusaka Adventist Eye Hospital between May and July 2013.

3.8 Data Collection Tool

Data information sheet was used as a tool to collect data from the respondents.

3.9 Data Collection Technique

Demographic data of patients (age, sex and ethnicity) was collected as per standard clinical practice and recorded on a provided laboratory form by the ophthalmic surgeon. An HIV result of patients was recorded by the researcher after reviewing patients' files. Data was collected over a period of 3 months.

3.10 Tissue Samples

Excision biopsies were taken after an informed consent by the ophthalmic surgeon as part of the routine clinical management of patients. The biopsy was divided into two pieces. One piece was fixed in 10% formalin and transported to the histology laboratory at UTH. The other piece was placed in a plain container and stored immediately in a deep freezer after excision and was later transported in a cool box

with ice blocks to UTH laboratory to be stored at -80^{o} C before being processed for HPV DNA extraction.

3.11 Tissue processing for histology

In the histology laboratory, tissues were received in 10% formalin and were grossed. Gross examination included describing the specimen and placing the tissue into a small plastic cassette. Initially, the cassettes were placed into a fixative (10% formalin).

After the tissue had been fixed, it was put into a tissue processor where it was paraffinised by running them through alcohols to xylenes and to wax for at least 18 hours. After removing them from the tissue processor, they were then properly oriented and put into blocks of paraffin.

After tissues had been blocked, they were cut using a microtome into 3µm sections and was placed on a slide and heated for 15 minutes.

Then slides were deparaffinised by running them through xylenes to alcohols and to water in that sequence. The slides were then stained with haematoxylin and eosin (H&E) as per attached protocol and examined to confirm the diagnosis. The slides were read by the principal investigator and the diagnosis was confirmed by the supervisor who is an experienced pathologist. The presence of a papillomatous growth pattern or koilocytosis was noted, and the lesions were classified as benign, conjunctival intraepithelial neoplasia (CIN) or invasive squamous cell malignancy as indicated in figure 1 on page 18.

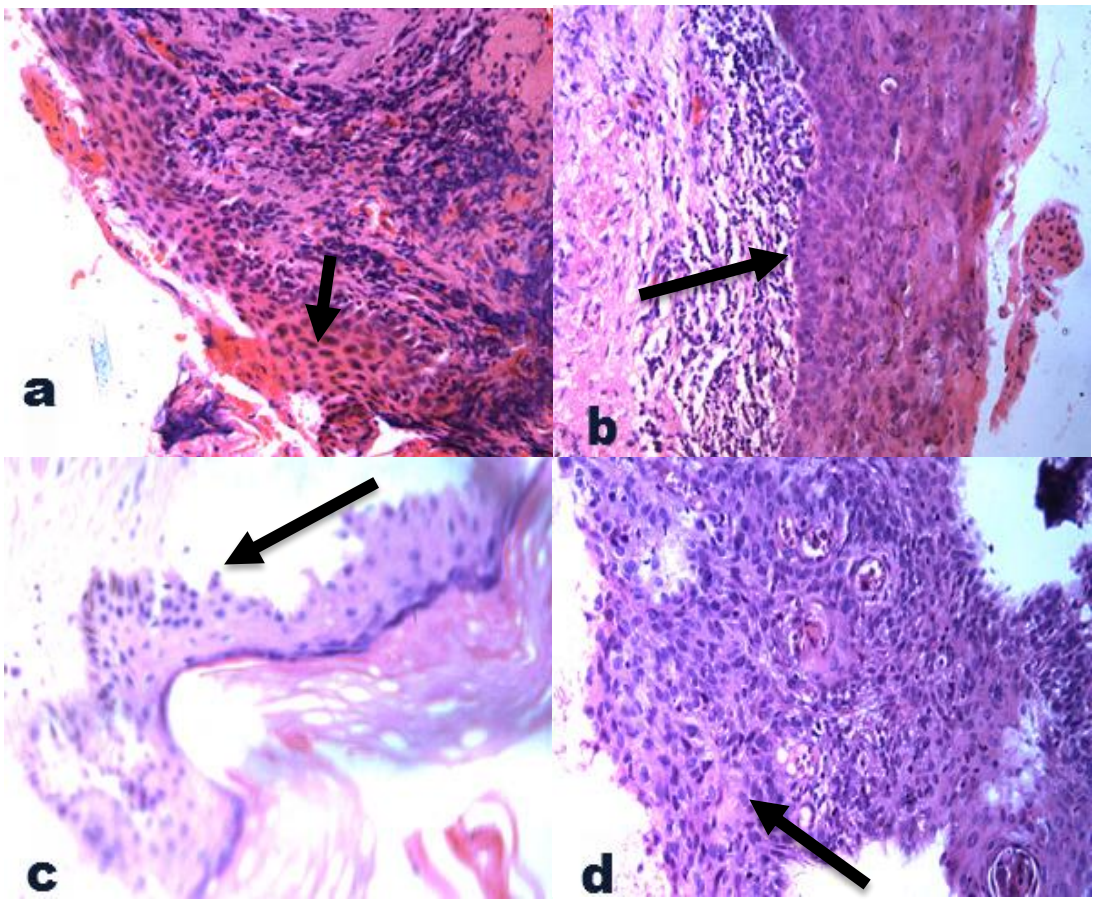

Figure 3.1: Micrographs of conjunctival lesions (a) Dysplasia, (b) Carcinoma in situ, (c) Micro invasive squamous cell carcinoma and (d) Moderately differentiated conjunctival squamous cell carcinoma.

3.12 HPV DNA extraction

DNA extraction was done with E.Z.N.A DNA extraction kit supplied by Omega Bio-tek, Inc. The frozen fresh tissue was allowed to thaw and 20-30 mg of tissue was then minced into small pieces and placed in a 1.5ml micro centrifuge tube. TL Buffer (200µl) was added to speed up the lysis. 25µl of reconstituted OB Protease was also added and vortexing was done to mix well the supernatant and later it was incubated in a shaking water bath set at 55°C overnight to effectively complete the lysis.

After lysis, the supernatant was centrifuged for 5 minutes at 13,000 x g to pellet insoluble tissue debris, later carefully aspirated and transferred to a sterile microfuge

tube. 220µl of BL Buffer was added and vortexed to mix. The supernatant was incubated at 70°C for 10 minutes then 220µl of absolute ethanol (room temperature, 96-100%) was added to the sample and mixed thoroughly by pipetting up and down for 5-10 times. The entire sample was transferred into the HiBind DNA Mini Column then centrifuged at 10,000 x g for 1 minute to bind DNA. After the centrifuge, the flow-through and collection tube was discarded.

The HiBind DNA Mini Column was inserted into a new 2 ml collection tube and 500 µl of HB Buffer was added and centrifuged at 10,000 x g for 30 seconds. The flow-through and collection tube was discarded and the HiBind DNA Mini Column was inserted into a new 2 ml collection tube. 700µl of DNA Wash Buffer (diluted with absolute ethanol) was added and centrifuged at 10,000 x g for 30 seconds. The flow-through liquid was discarded and the HiBind DNA Mini Column inserted back into the same collection tube. 700 µl of DNA Wash Buffer was added and centrifuge at 10,000 x g for 30 Seconds and the flow-through liquid was discarded and the HiBind DNA Mini Column was placed back into the same collection tube and centrifuged at a maximum speed of (>13,000 x g) for 2 minutes to dry the column.

The HiBind DNA Mini Column was inserted into a sterile 1.5 ml microfuge tube, 100-200µl of preheated (70°C) Elution Buffer (10mM Tris, pH8.5) was added and allowed to sit at room temperature for 2 minutes then DNA was eluted from the HiBind DNA Mini Column by centrifuging at 13,000 x g for 1 minute.

After the extraction of DNA, the elution was tested for the presence of DNA by gel electrophoresis and all of them were DNA positive as indicated in figure 2 below.

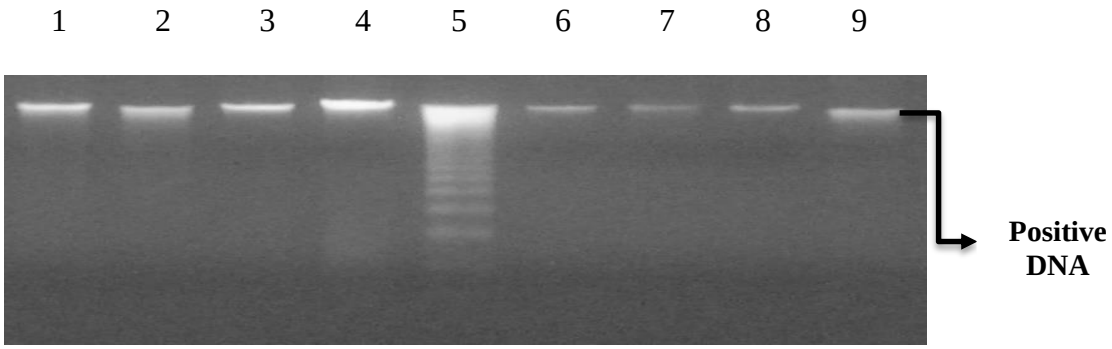

Figure 3.2: Gel Electrophoresis of DNA products on 2% agarose gel. The presence of a DNA band on the gel was recorded as positive and the absence of the band as negative.

3.13 Polymerase Chain Reaction Assay

PCR was performed with the universally accepted consensus primers MY9/MY11 supplied by Inqaba Biotechnical Industries (Pty) Ltd, in South Africa. These primers amplified the majority of the known HPV types 2A, 6, 7, 8, 9, 10, 11, 13, 15, 16, 17, 18, 21, 22, 23, 24, 25,28, 29, 31, 32, 33, 34, 35, 36, 38, 40, 42, 44,45, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 59,61, 62, 63, 64, 66, 67, 68, 69, 70, 72, 73.

The extracted DNA (3.0µl) was mixed with 1.0ul of ddH_2O, 25µl of PCR master mix, 0.5µl of MY 9/MY 11 primers each with appropriate positive and negative controls targeting a 288 bps fragment of the single copy gene.

The sample was considered HPV positive when a DNA sample of a predicted size was identified. It was considered negative when a DNA sample of a predicted size was not identified as indicated in figure 3 on page 21.

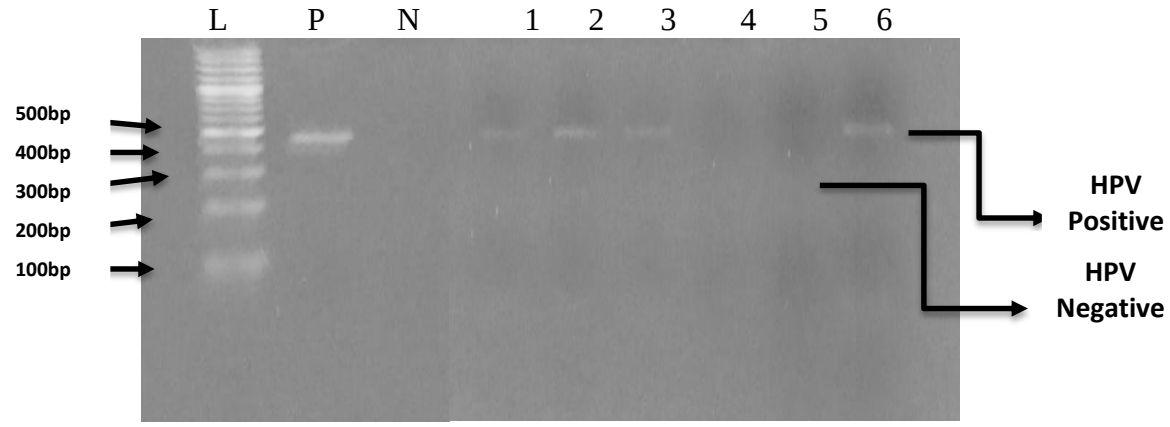

Figure 3.3: Amplification of HPV DNA with consensus primers MY9/MY11 on 2% agarose gel. L - Ladder (100bp marker), P - Positive control, N – Negative control (nuclease free water), Lane 1 to 6 – viral DNA of HPV.

In addition, 7 percent of all samples were randomly subjected to a repeat PCR test to determine the reproducibility of the results.

3.14 HIV test

All patients who underwent surgery were tested for HIV as per standard hospital clinical practice. Therefore, HIV result was obtained from the patients' file.

3.15 Data analysis

Data was entered into SPSS version 18.0 for analysis. Univariate analysis of variables, which were CSCC, HPV and HIV was conducted to determine the distribution patterns. The association of HPV to CSCC and HPV to HIV was calculated using Odds ratio.

3.16 Reliability

The same data information sheet was used to collect data from all the respondents. It was also maintained by ensuring that a separate microtome blade was used for each tissue to avoid contamination and all good laboratory practices were followed. It was

also maintained by using primers from a reputable scientific company - Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

3.17 Validity

Validity was maintained by ensuring that all variables under study were covered in the data information sheet. These variables included HIV, HPV and CSCC. HIV was recorded positive if it was reactive to the antibodies detecting HIV and was recorded negative if it was non-reactive. HPV was considered positive when a DNA sample of a predicted size was identified on agarose gel electrophoresis and negative when it is not identified. The lesion was considered benign or malignant based on the presence of papillomatous growth pattern or koilocytosis.

3.18 Ethical Considerations

Ethical clearance was obtained from the University of Zambia Biomedical Research Ethics Committee (UNZABREC). Permission to conduct the study was obtained from the Senior Medical Superintendent at UTH and the Medical Director at Lusaka Adventists Eye Hospital.

Written informed consent was also obtained from participants. They were assured that, the information was to be used for research purposes only and their participation was voluntary.

Anonymity and confidentiality was maintained by ensuring that no name but only serial numbers appeared on the data information sheet and tissue samples. This also applied to the time when data analysis was being done and all used data information sheets were stored under lock and key for future references . No one had access to the completed data sheet apart from the researcher.

Participants were further reassured that the study did not put them at risk as the investigator only used tissue samples which were removed by their health care providers as part of their treatment.

CHAPTER 4: RESULTS AND DISCUSION

4.1 RESULTS

This section presents results that firstly indicate the prevalence of HPV in CSCC and secondly, the results that show the association of HPV and HIV infections with CSCC. Lastly, they present the co-existence of HPV infection and HIV infection in CSCC.

TABLE 4.1: CHARACTERISTICS OF PATIENTS WITH CONJUNCTIVAL SQUAMOUS CELL CARCINOMA (N=37)

Variable		Frequency	Percent
Health Institution	University Teaching Hospital	10	27.0
	Lusaka Eye Hospital	27	73.0
	Total	37	100.0
Sex	Male	17	45.9
	Female	20	54.1
	Total	37	100.0
Age	20 - 45	31	83.8
	46 and above	6	16.2
	Total	37	100.0
Race	Black	37	100.0
Country	Zambia	37	100.0
HIV Status	HIV Positive	21	56.8
	HIV Negative	16	43.2
	Total	37	100.0

Table 4.1 shows that, most of the conjunctival lesions (n=27, 73%) came from Lusaka Eye Hospital. Most of the patients (n=20, 54.1%) were females and (n=31, 83.8%) were below the age of 45 years. All the participants were indigenous black Zambians and (n=21, 57%) were HIV positive.

TABLE 4.2: DESCRIPTIVE STATISTICS FOR LABORATORY FINDINGS (N=37)

Variable		Frequency	Percent
Histology Results	Malignant Tumours	22	59.5
	Benign Tumours	15	40.5
	Total	37	100
Histological Classifications of Conjunctival Lesions	Hyperkeratosis	3	8.1
	Dysplasia	6	16.2
	Hyperplasia	5	13.5
	Capillary Haemangioma	1	2.7
	Kaposi's Sarcoma	1	2.7
	Carcinoma in Situ	1	2.7
	Keratinizing SCC	5	13.5
	Invasive SCC	15	40.5
	Total	37	100.0
Malignant Tumours	CSCC*	21	95.5
	Kaposi's Sarcoma	1	4.5
	Total	22	100.0
Groups	CSCC	21	56.8
	Non-CSCC**	16	43.2
	Total	37	100.0
PCR Results	HPV Positive	21	56.8
	HPV Negative	16	43.2
	Total	37	100.0

Invasive SCC, Keratinizing SCC and Carcinoma in Situ, ** Hyperkeratosis, Dysplasia, Kaposi's sarcoma, Capillary Haemangioma and hyperplasia

Table 4.2 shows that, most of the conjunctival lesions (n=22, 59.5%) were malignant of which CSCC (n=21/22, 95.5%) was the most prevalent while (n=1, 4.5%) were Kaposi's sarcomas. HPV DNA was detected in (n=21, 56.8%) of the conjunctival lesions.

CROSS TABULATIONS

TABLE 4.3: PREVALENCE AND ASSOCIATION OF HPV AND HIV INFECTIONS WITH CSCC (N=37)

Variable		Cases		Total	Unadjusted OR	Adjusted OR	P value
		CSCC	Non-CSCC				
SEX	Male	12 (57.1%)	5 (31.2%)	17 (45.9%)	2.933	.365	.253
	Female	9 (42.9%)	11 (68.8%)	20 (54.1%)	(95% CI 0.749 - 11.491)	(95% CI .065 - 2.056)	
	Total	21 (100%)	16 (100%)	37 (100.0%)			
AGE	20 - 45	19 (90.5%)	12 (75%)	31 (83.8%)	3.167	.269	.255
	46 and above	2 (9.5%)	4 (25%)	6 (16.2%)	(95% CI 0.500 - 20.036)	(95% CI .028-2.578)	
	Total	21 (100%)	16 (100%)	37 (100%)			
HIV Status	HIV Positive	15 (71.4%)	6 (37.5%)	21 (56.8%)	4.167	.158	.048
	HIV Negative	6 (28.6%)	10 (62.5%)	16 (43.2%)	(95% CI 1.042 - 16.660)	(95% CI .025-.986)	
	Total	21 (100%)	16 (100%)	37 (100%)			
PCR Results	HPV Positive	15 (71.4%)	6 (37.5%)	21 (56.8%)	4.167	.137	.032
	HPV Negative	6 (28.6%)	10 (62.5%)	16 (43.2%)	(95% CI 1.042 - 16.660)	(95% CI .022-.840)	
	Total	21 (100%)	16 (100%)	37 ((100%)			

CSCC was not associated with sex (OR 2.933, 95% CI 0.749 - 11.491) or age category (OR 3.167, 95% CI 0.500 - 20.036). However, CSCC was associated with HIV status (unadjusted OR: 4.167, 95% CI 1.042 - 16.660) and HPV (unadjusted OR: 4.167, 95% CI 1.042 - 16.660) as seen in table 4.3.

Table 4.4: CO-EXISTENCE OF HPV INFECTION WITH HIV INFECTION (N=37)

		PCR Results		Total	Unadjusted
		HPV +	**HPV -**		**OR**
HIV	HIV +	11 (52.4%)	10 (47.6%)	21 (100.0%)	0.660
Status	HIV -	10 (62.5%)	6 (37.5%)	16 (100.0%)	(0.175 - 2.485)
	Total	**21 (56.8%)**	**16 (43.2%)**	**37 (100.0%)**	

There was no association between HIV status and HPV status (OR 0.660, 95% CI 0.175-2.485) as seen in table 4.4.

4.2 DISCUSSION

This study set out to establish the prevalence and association of HPV infection with CSCC at UTH and Lusaka Eye Hospital in Zambia. It further assessed the excess of HPV infection in patients with HIV.

Seventy three percent of the participants came from Lusaka Eye Hospital of which 54.1% were females and 83.8% were between 20 - 45 years old. All the participants were indigenous black Zambians.

A total number of 37 conjunctival tissue specimens suspected to be CSCC were histologically examined with a light microscope. Lesions ranged from dysplasias to invasive squamous cell carcinomas. The study findings showed that, 59.5% of the conjunctival lesions were malignant and among the malignant tumours, 95.5% were CSCC (comprising of carcinoma in situ, keratinizing squamous cell carcinoma and non-keratinizing invasive squamous cell carcinoma) and 4.5% were Kaposi's sarcoma. Benign tumours included hyperkeratosis, dysplasia, haemangioma and hyperplasias. In this study, no papilloma was observed.

The results of this study revealed that, CSCC was more common in males (57.1%). These findings agree with Ali Baig et al., (2009) in a study entitled squamous cell carcinoma of the conjunctiva: analysis of fifteen cases conducted in Pakistan who reported high CSCC in males. However, in our study, the difference was minimal as compared to Ali Baig et al., (2009) who reported 80% of CSCC in males. The difference could have been due to high HPV and HIV infections in our study population affecting both male and females equally compared to 0.557% prevalence of HIV in Pakistan (Muhammad, 2014). It was also observed that, 90.5% of CSCC was in patients aged below 45 years. Findings in this study are in contrast to those by

Baig et al., (2009) who reported high numbers of CSCC in the age group ranging between 63-87 years. These differences might be due to a low prevalence of AIDS in Pakistan (Muhammad, 2014). However, the findings in this study are consistent with those by Ateenyi-Agaba, (1995) and Waddell et al, (1996). In their studies, CSCC was more common in the young age groups ranging from 20 to 40 years. The consistence may be due to similarities in factors affecting Zambians and Malawians such as high prevalence of HIV.

Polymerase chain reaction assay was performed to determine the presence of HPV in conjunctival squamous cell carcinomas. Twenty one CSCC and 16 control conjunctival lesions were tested for the presence of HPV DNA. Amplified specific HPV DNA was detected in 71.4% CSCC and 37.5% non-CSCC (unadjusted OR: 4.167, 95% CI 1.042 - 16.660). Since HPV is commonly transmitted through sexual intercourse, its detection in conjunctival lesions maybe due to self-inoculation. These results are in agreement with Amoli, et al. (2011), who detected HPV DNA in 92% samples of squamous cell carcinoma in Iran. This also accords with other earlier observations that, conjunctival squamous cell carcinomas are associated with HPV infection (Nakamura, et al., 1997; Karcioglu and Issa, 1997). It can thus be suggested that, HPV infection has a role in the aetiology of conjunctival CSCC in the Zambian population and is responsible for most of the CSCC occurring in young adults.

This study also revealed that, 71.4% of CSCC was in HIV positive patients (unadjusted OR: 4.167, 95% CI 1.042 - 16.660) compared to controls 37.5%. These results suggest that, HIV have a role in CSCC in indigenous Zambians. The findings agree with Gichuhi et al., (2013) in a study "Epidemiology of Ocular Surface Squamous Neoplasia (OSSN) in Africa" were HIV (OR = 6.17, 95% CI: 4.83-7.89) was strongly

associated with OSSN especially in Southern Africa. Zambia is one of the countries in Southern Africa and these results cannot be a surprise. Waddell et al, (1996) also found similar results in a study entitled carcinoma of the conjunctiva and HIV infection in Uganda and Malawi. This study's findings also agrees with the findings of Spitizer, et al., (2008), in a study, OSSN as the first apparent manifestation of HIV infection in Malawi. Spitizer found that, of all the patients with malignant ocular tumours, 80% (39 of 49) had CSCC, and 76% of the patients with CSCC were HIV positive. CSCC has become the most frequent malignant ocular tumour in Zambia and the incidence seems to have increased in parallel to increasing HIV infection rates.

The results of this study show that, HPV DNA was detected in 52.4% of HIV positive patients and 62.5% of HIV negative patients (OR 0.660, 95% CI 0.175 - 2.485). Although HPV was detected in HIV patients in this study, the observed difference was not significant. In contrast to earlier findings of Luchters, et al. (2010) and Smith, et al. (2010), no co-existence of HPV infection was established in patients with HIV. Luchters, et al. (2010) reported four fold of HPV infections in HIV patient. A possible explanation for this might be that, earlier studies focused on sex workers who were vulnerable to both HIV and HPV infections. Another reason could have been that, earlier studies concentrated on genital HPV infection rather than general HPV infection in relation to HIV acquisition in women.

4.1. Limitations of the study

Due to resource constraints, this study did not characterize human papilloma viruses. However, this project provided an important opportunity to advance the understanding of HPV and HIV in relation to CSCC in indigenous Zambians.

The author suggests that further investigation into the distribution of HPV types among various populations will shed more light on links between HPV and CSCC.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

To the best knowledge of the researcher, this is the first study to report on the association of HPV with CSCC in the indigenous black Zambians.

The study showed a high prevalence of HPV infection (71.4%) in CSCC. HPV and HIV infections were both associated with CSCC. Though HPV infection was found in patients with HIV, there was no difference in the occurrence of HPV in HIV positive and HIV negative patients.

5.2 Recommendations

- Due to a small sample size, these findings might not be generalizable to all indigenous Zambians. Therefore, further studies with larger samples on the current topic are recommended.
- To investigate the genotypes of the most prevalent HPV is required. This would lead to the development of vaccine for acquired ocular lesions.

5.3 Dissemination and utilization of findings

The findings of the study were presented to the Department of Pathology and Microbiology, School of Medicine, UNZA. Then, the results were later presented at the postgraduate seminar week. The results will be also presented to various stake holders involved in the management of eye diseases at various fora such as conferences.

UTH and Lusaka Eye Hospital which were the study sites will be given a copy of the study results report so that these hospitals would use them to further their knowledge on the risk factors for CSCC.

In addition, four copies of the research report will be printed and submitted to the following;

1. Department of Pathology and Microbiology
2. UNZA Medical Library and Main Library
3. Ministry of Health
4. Researcher

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Appendix 1.

PATIENT DATA INFORMATION SHEET

CHARACTERIZATION OF HUMAN PAPILLOMA VIRUSES IN CONJUNCTIVAL SQUAMOUS CELL CARCINOMA AT THE UNIVERSITY TEACHING HOSPITAL, ZAMBIA

SERIAL NUMBER: .....

DATE: .....

NAME OF HEALTH CENTRE: .....

INSTRUCTIONS

1. No name should appear on/and or in this questionnaire.
2. Answer all the questions.
3. Put the letter "X" in the box next to your choice.
4. Use a pen/pencil in the questionnaire.
5. Information given in this questionnaire will be kept confidential.

FOR OFFICIAL USE ONLY

SECTION A: BACKGROUND INFORMATION

1. Age

2. Sex
   a) Male
   b) Female
3. Ethnicity
   a) Black
   b) Indian
   c) Chinese
   d) Other – Specify …….
4. Country
   a) Zambian
   b) Other – specify …….

SECTION B:

5. HIV status
   a) Positive
   b) Negative

6. Any genital or skin lesion suspected to be HPV?
   a) Yes
   b) No

SECTION C: LABORATORY RESULTS

7. Histology results
   a) Benign
   b) CIN
   c) Malignant

8. PCR (HPV) Results
   a) Positive
   b) Negative

Appendix 2.

INFORMATION SHEET FOR THE PATIENT

TITLE OF THE STUDY:

CHARACTERIZATION OF HUMAN PAPILLOMA VIRUS IN BENIGN AND MALIGNANT CONJUNCTIVAL LESIONS CLINICALLY SUSPECTED OF SQUAMOUS CELL CARCINOMA AT THE UNIVERSITY TEACHING HOSPITAL, ZAMBIA

This study involves examining the growth growing on your eye which will be removed by your Doctor as part of your treatment. The examination will be under the microscope to see if the growth has cancer cells or not as per your Doctor's request and an additional test (PCR) will be conducted to find out if the growth harbors human papilloma virus or not. The purpose of this study is to help health workers improve on the current treatment and prevention strategies concerning cancer of the eye. All examinations on the growth growing on your eye (microscopic and PCR) will be conducted by the researcher.

Your participation in this research is entirely voluntary which means you do not have to participate if you do not wish to. Refusal to take part will involve no penalty or loss of services to which you are entitled to. If you decide to take part, you are still free to withdraw at any time without penalty or loss of services and without giving any reason for your withdrawal.

Participating in this study does not put you at any risk at all as it involves only examining a conjunctival lesion which has been removed by your Doctor as part of treatment for your condition. You are going to benefit in this study by not paying your laboratory fees for these tests while in a normal circumstance you are required to pay.

As a participant, you may choose not to answer particular questions that are asked in the study. If there is anything that you would prefer not to discuss, please feel free to say so. The information collected in this interview will be kept strictly confidential.

If you have any question about the study please contact the principal investigator at the following numbers; 0966-560958, 0975-214406 or write to an email chapima2007@yahoo.com

You can also contact the Biomedical Research Ethics Committee at the following addresses,

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@zamtel.zm

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

If you choose to participate in this research study, please sign an informed consent form below.

Appendix 3. TRANSLATED INFORMATION SHEET

IFYO MUFWILE UKWISHIBA PALI UKU KUFWAILISHA KWA BULWELE BWA CANCER YAMENSO

UKUFWAILISHA KWA MITUNDU YA TUSHISHI UTUSANGWA MULI CANCER YA MUMENSO PA UTH MU ZAMBIA

Uku kufwailisha kwa mitundu ya tushishi utusangwa muli cancer ya mumenso, kule fwaikwa ukupima akamunofu akaamena palinso lyenu ukumona ngacakuti nakakwata cancer elyo nokufwailisha utushishi utungalenga ututuminofu ukumena. Bashinganga abalemimona bali no ku micencenta bwino bwino nokumona ngacakuti akamunofu akaamena palinso lyenu ni cancer nangula iiyo. Nga batunganya ati ni cancer ninshi bali nokumibombela pakuti ba kafumye aka kamunofu. Ngabafumya akamunofu, nga cakuti mwansuminisha ndefwaya ukukapima munshila shibili. Inshila yakubalilapo, kwishiba ngacakuti aka kamunofu nakakwata cancer ngefyo bashinganga benu baletunganya nangu iyo. Ne cabubili kufwailisha akashishi akanga leta ubu ubwafya.

Ukuibimba muli uku kufwailisha kwaubu bulwele kuitemenua fye. Tekupatikishiwa iyo. Ngacakweba ati tamulefwaya ukuibimbamo temulandu iyo bashinganga bakamitangata fye bwino bwio ukwabula akapatulalula. Ngacakweba ati pakubala mwaibimbamo elyo mulefwaya ukulekela panshila nacena cili fye bwino. Kuti mwaleka ukwabula ukulondola ico mwalekela ukwabula ubwafya.

Ukuibimba muli uku kufwailisha kwa ubu bulwele tamuleibika mubusanso nangu bumo pantu abale fwailisha pa lwa ubu bulwele balesendako fye akaamunofu ako bashinganga wenu bafumishe palinso lyenu ukulingana nobundapishi nokukatuma ku labu uko kalefwaikwa ukukapima. Kuti mwakwatilamo ubunonshi muli uku kufwailisha kwa ubu bulwele pantu tamwakalipilepo ulipiya nangu lumo nge fyo ciba pakupima aka kamunofu pakukamona nga nakakwata cancer nangu iyo.

Uku kufwailisha kwa ubu bulwele kuli ukwayana pantu kule afwilisha bashinga ukwishiba ifingi pali ubu bulwele pakuti baleundapa bwino abo abalesangwa nabo. Abakapima ifi fyonse ni ababene abalefwailisha pali ubu bulwele. Ngamulefwaya ukwishiba ifyo tukasanga ngatwapima, bashinga wenu abalemimona kuti ba myeba.

Ishibeni ukuti, ukulandashanya konse twalakwata na mepusho yonse fyankama. Ngacakweba ati na mwasumina ukuibimbamo saineni aka pepala.

Appendix 4.

VOLUNTARY CONSENT

I have read (or have had explained to me) the information about this research as contained in the Participant Information Sheet. I have had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction.

I now consent voluntarily to be a participant in this project and understand that I have the right to end the interview at any time, and to choose not to answer particular questions that are asked in the study.

My signature below signifies that I am willing to participate in this research:

Participant's name (Printed): …….....……

Participant's signature: …….…… Consent Date: ……

Researcher Conducting Informed Consent (Printed) ……

Signature of Researcher: ……..……. Date: …….……

Witness: ……..…….

Signature: ……..…… Date: ……

Thumb print …….

Appendix 5: H&E TECHNIQUE PROTOCOL

SOP TITLE: HAEMATOXYLIN AND EOSIN TECHNIQUE
SOP NO.: HISTO 04
YEAR REVIEWED: 2008

Purpose
The technique is used to demonstrate the general tissue morphology and serves as the main diagnostic staining method.

Principle
Haematoxylin a dark purplish positively charged dye stains chromatin within the nucleus, while eosin, a pink to red negatively charged dye stains cytoplasmic elements, connective tissue and muscle in various shades of red, highlighting the general tissue structure.

Equipment and Materials
- Staining troughs
- staining rack
- Slides
- Coverslips
- Mounting medium

Preparation of reagents: refer to Appendix No. D1

Storage and Stability
- **Reagents**
  - Ehrlich's haematoxylin – should be stored in an amber bottle and away from sunlight. Solution is stable for up to 5 years.
  - 1% aqueous eosin: stable up to one year.
  - 1% acid alcohol: Prepare monthly.
  - Scott's tap water substitute: keeps up to six months.
  - Xylene: store in metal cabinets.
  - Absolute alcohol: store in metal cabinets.
- **Kits:** Not applicable
- **Specimen Type and Collection:** 5 µm paraffin processed sections
- **Handling**: Wear gloves, goggles and lab coat. Work under fume hood, avoid contact and inhalation.

Calibration
Not applicable

Quality Control
Apply a drop of haematoxylin to a filter paper and note for appearance of purple border. Absence of purple implies an expired solution. Alternatively dispense a drop of haematoxylin into a beaker containing tap water and note for change of colour from red to blue. If the red colour persists, then the haematoxylin should be discarded.

Procedure

1. Take sections to water, that is deparaffinise in two changes of xylene (2minutes each), wash in 2 or 3 changes of absolute alcohol (2 minutes each) then in water for 2 minutes.
2. Stain with Ehrlich's haematoxylin solution for 25 minutes.
3. Wash in running tap water for 5 minutes.
4. Differentiate in 1% hydrochloric acid in 70% alcohol (1% acid alcohol) for 15-30 seconds.
5. Blue in running tap water for 10 minutes or in Scott's tap water substitute for 1 minute.
6. If the bluing agent has been used, rinse in tap water briefly (10 dips).
7. Counter stain in 1% aqueous eosin for 2-5 minutes.
8. Rinse in 95% alcohol (10 dips).
9. Dehydrate in 2 changes of absolute alcohol for 2 minutes in each, clear in 2 or 3 changes of xylene for 2 minutes each.
10. Mount in DPX.

Calculations

Not applicable

Results

Nuclei – Dark blue
Cytoplasm, connective tissues, muscle, and any acidophil granules - pink
Red blood cells - red.

Interpretation of results

The nucleus, cytoplasm and other tissue structures are made visible with two or more contrasting colours bringing out the morphological characteristics which aid tissue identification

Limitations of the Procedure and Sources of Error

- Overoxidised haematoxylin or understaining will yield pale stained nuclei.
- Eosin solution must be between pH 4.0 and 5.0 because If the pH is too low, the eosin will convert to free acid, dominate the solution and yield a muddy stain. If it is too high, the net charge on the protein will become negative and it will have no affinity for the dye.

Disposal

- Reagents: pour down the drain with copious volumes of water.
- Specimen: all remnants of tissue must be incinerated.
- Waste: xylene must be collected in bottles for specialist disposal.

References

Crookham, J, Dapson, R, (1991), Hazardous Chemicals in the Histopathology Laboratory, 2nd Ed, Anatech LTD

Gamble M, Bancroft J.D, (2002) Theory and Practice of Histological Techniques, 5th Ed, , Churchill
Livingstone, NY, pp 125-131

Appendix 6: AUTHORITY LETTER TO CONDUCT A STUDY

THE UNIVERSITY OF ZAMBIA

SCHOOL OF MEDICINE

Telephone: +0211-252641
(Pre-Clinical) Ridgeway Campus
Telegram: UNZA, Lusaka
Telex: UNZALUZA 44370
Fax: +260-1-250753

Department of Pathology & Microbiology
P.O. Box 50110
Lusaka, Zambia

Your Ref:
Our Ref:

14th September, 2012

The Senior Medical Superintendent
University Teaching Hospital
P.O. Box RW 1X
LUSAKA

Dear Sir,

Re: **REQUEST FOR PERMISSION TO CONDUCT A RESEARCH STUDY**

This is to introduce Mr Chapima a Master of Science in Pathology (Clinical Pathology) student. It is the programme's requirement that MSc students do their final year research project in line with their field of study to graduate. It is to this effect that the School of Medicine recommends the above candidate to you for her research and data collection.

In partial fulfillment of this program, he is required to conduct a research study and his topic is **"Prevalence of human papilloma virus in benign and malignant conjunctival tissues clinically suspected of squamous cell carcinoma at the University Teaching Hospital, Zambia".**

I write to request your good office to allow him use the conjunctival tissues clinically from your institution.

Your support in this matter will be highly appreciated.

Yours faithfully,

Dr T. Kaile
HEAD OF DEPARTMENT OF PATHOLOGY AND MICROBIOLOGY

Appendix 7: UNZABREC CLEARANCE LETTER

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

1st February, 2013.

Your Ref: 006-10-12.

Mr. Chapima Fabian,
School of Medicine,
Department of Pathology & Microbiology
PO Box 50110,
Lusaka.

Dear Mr. Chapima,

RE: RE-SUBMITTED RESEARCH PROPOSAL: **"CHARACTERIZATION OF HUMAN PAPILLOMA VIRUS IN BENIGN AND MALIGNANT CONJUCTIVAL LESIONS CLINICALLY SUSPECTED OF SQUAMOUS CELL CARCINOMA AT THE UNIVERSITY TEACHING HOSPITAL, ZAMBIA" REF. NO 006-10-12.**

The above mentioned research proposal was resubmitted to the Biomedical Research Ethics Committee with recommended changes on 1st February, 2013. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C Munthali
CHAIRPERSON

Date of approval: 1st February, 2013 **Date of expiry**: 31st January, 2014