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HUMAN PAPILLOMAVIRUS GENOTYPE CONCORDANCE WITHIN ZAMBIAN COUPLES

By

Kgomotso Makhaola

A THESIS

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HUMAN PAPILLOMAVIRUS GENOTYPE CONCORDANCE WITHIN ZAMBIAN COUPLES

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University of Nebraska, 2011

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Abstract: Human Papillomavirus (HPV) is strongly associated with the development of cervical cancer, and has also been identified in other anogenital cancers such as penile, oral, and anal cancers. In regions like Sub Saharan Africa where the prevalence of HIV/AIDS is high, increased rates of HPV infections have also been observed, however the effect of HIV/AIDS on the transmission of HPV is not yet well understood. In this study specimens for HIV, HPV testing and pathology were collected from male participants and their female partners at a Urology department in Lusaka University Teaching Hospital, Zambia. Seventy four samples were collected but only thirty four (46%) yielded DNA for HPV testing. HPV detection was done with GP5+/GP6+ PCR, followed by DNA sequencing; and HIV detection was done by serology. HPV 16 specific primers were used for L1 and LCR variants identification. RESULTS: The overall HIV Prevalence was 78.6%, and HPV prevalence among the HIV positive was 68% compared to 30% amongst the HIV negative participants. HPV genotype concordance was observed in 7(43.8%) couples tested for HPV, and amongst these only 6 had sequence similarity.HIV concordance among these HPV concordant couples was 67%.However, the role of HIV on HPV type concordance could not be established due to small sample size. Variants of HPV 16 in L1 and LCR region were identified. Six novel HPV 16 variants with nucleotide exchanges in the LCR region were identified. Five were classified as the HPV 16 variant European group, and one as the African group. Two of these variants had relatively lower promoter activity, 30% of that of the wild type strain. The decreased promoter activity of some HPV 16 variants decreases expression of viral oncogenes and may be linked with the development, phenotype and prognosis of the cervical lesions in women infected with these kinds of HPV 16 variants.

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CHAPTER I

LITERATURE REVIEW

Biology of Human Papillomaviruses

Human Papillomavirus is a small, circular double stranded DNA virus that infects cutaneous and mucosal keratinocytes [Graham. 2010]. More than 120 HPV genotypes have been identified and categorized based on DNA sequence analysis [de Villiers et al. 2004]. At least 40 mucosal genotypes infect the genital tract of which 15 types (HPV 16, -18,-31,-33,-35,-39,-45,-51,-52,-58,-59,-66, and -68) are categorized as high risk (HR-HPV) based on their oncogenic potential. The other mucosal HPV types (HPV 6,-11,-40,-44,-42,-43,-54,-61,-72 and -81 fall in the low risk group and these are associated with warts and or low grade intraepithelial lesions [Miura et al. 2010] .

The prototype genome, HPV 16 is 7904bp and it has three regions, Locus Control Region (LCR), the Early genes region and the Late genes region (Figure 1.1). The LCR is a non-coding region that functions as the major regulatory unit for gene expression. It contains a keratin dependent promoter and the origin of replication [Graham. 2010]. The Early Open Reading Frames (ORFs) encode proteins expressed early in viral infection and these are the transforming proteins E6, E7 and E5; the replication proteins E1, E2 and viral assembly protein E4. The late ORFs encode L1, a major capsid protein and L2 which is a minor capsid protein and these are expressed in highly differentiated cells late in infection. These capsid proteins are also important for viral entry into the cell [Graham. 2010, McLaughlin-Drubin et al. 2011].

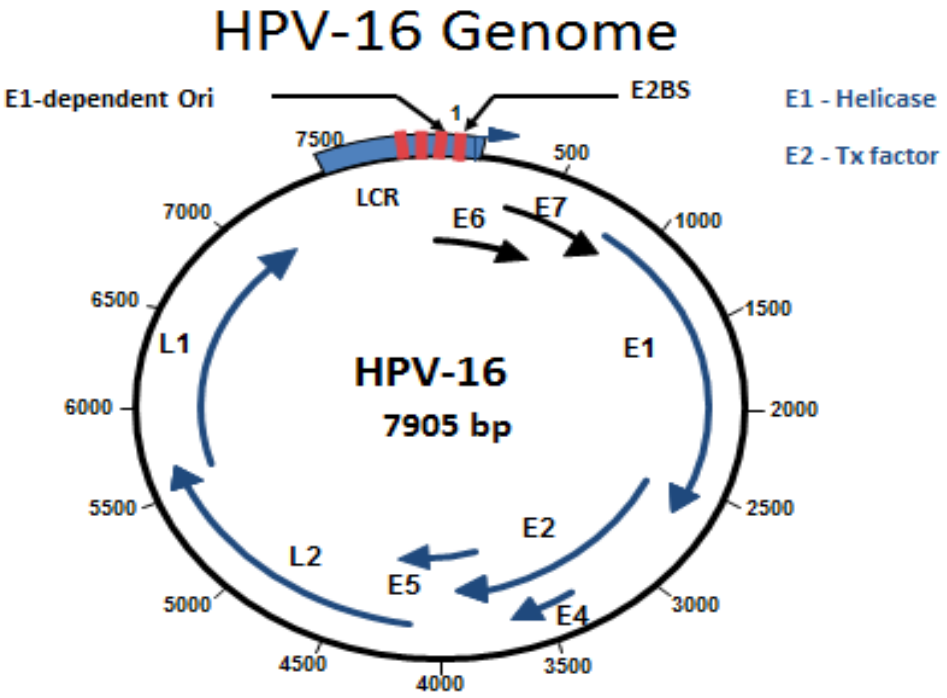


Figure 1.1 Human Papillomavirus (HPV) 16 genome. (E1-E7 early genes, L1-L2 late genes: capsid)

HPV Life cycle

HPV has tropism for keratinocytes and the initial HPV infection occurs in the basal epithelial cells through an abrasion or cut in the epithelial layer (Figure 1.2). While specific cell entry receptors have not been identified, it is speculated that HPV uses heparin receptors and enters the cell through claritin coated vesicles [Graham. 2010]. Once inside the cell, HPV replicates extra-chromosomally with the help of cellular enzymes. HR-HPVs have well established mechanisms for transforming cells. They modify the expression activity of many cellular proteins in order to promote cell proliferation; inhibit apoptosis and decrease cell differentiation [McCloskey et al. 2010, McLaughlin-Drubin et al. 2011]. They also have mechanisms to suppress the immune

system to enable amplification of the viral genome. Oncogenic proteins HR-HPV E6, E7 and E5 are expressed early during infection in the basal layer which contains stem cells. The HPV genome is amplified and shared amongst daughter cells providing a pool of HPV genome for every cycle of cell division. The HR-HPV E6 through binding the E6AP which is a ubiquitin ligase degrades p53 in a proteasome dependent manner [Jha et al. 2010, McLaughlin-Drubin et al. 2011] inhibiting apoptosis or G1 cell cycle arrest. HR-HPV forces cells to enter into S phase by expressing HR-HPV E7 that binds the

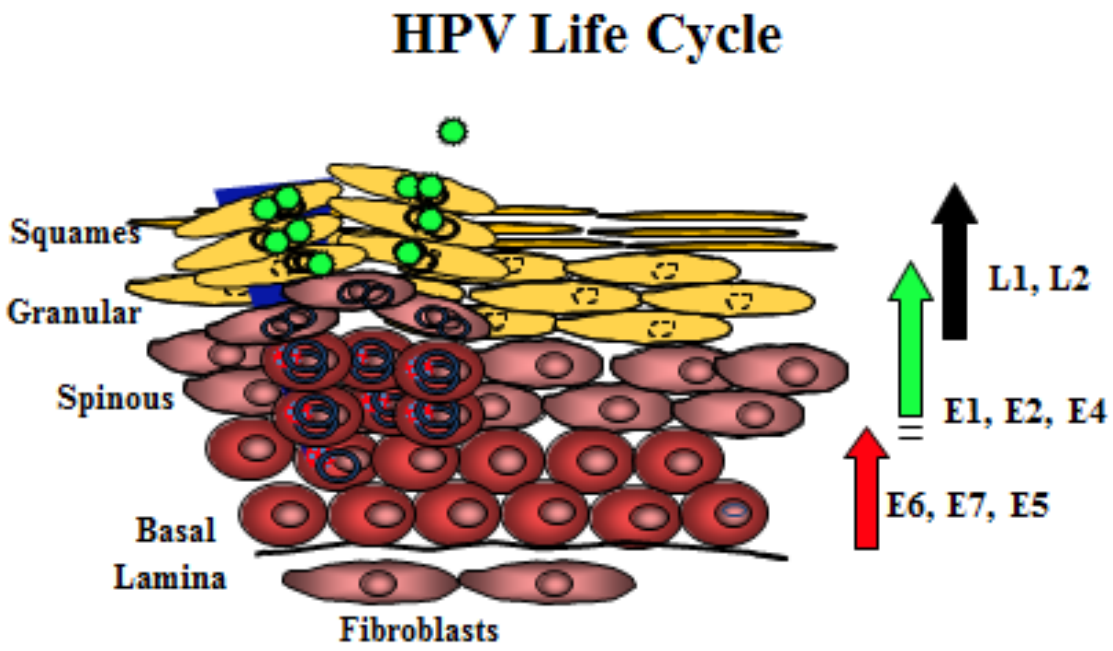


Figure 1.2 HPV Life cycle: Early Proteins E6, E7 and E5 are expressed in the basal cells and as the epithelial cells differentiate the replication proteins E2, E1 and viral assembly protein, E4 are expressed. The late proteins L1 and L2 are expressed in fully differentiated cells.

hypo-phosphorylated pRb [Banerjee et al. 2011]. Rb family of proteins interact with E2F family of proteins at physiological levels to regulate cell cycle. Binding of HR-HPV E7 to pRb releases E2F which activates the DNA synthesis machinery [Chellappan et al.

1991]. The expression level of HR-HPV E7 is closely correlated with the transforming potential of the virus, and high levels of HR-HPV E7 mRNA expression have been found in oral and cervical cancer biopsy samples [Jha et al. 2010]. The other mechanism that HR-HPV E7 uses to maintain viral DNA is by extending the G2 phase through the induction of cyclin B1 and cdc2 that accumulate in the cytoplasm of suprabasal cells [Banerjee et al. 2011]. McCloskey, R et al have also shown that HR-HPV E7 increases cellular proliferation and decrease proliferation through increased expression of nucleophosmin nucleolar protein, allowing for increased transcription of viral genome. [McCloskey et al. 2010].

In order to evade the immune system HR-HPV E7 down regulates the expression of MHC I on the infected cells to avoid immune surveillance and killing by CD8+Cytolytic cells [Heller et al. 2011]. The other protein that enhances transformation of cells by HR-HPV is the HPV E5. A study showed that mice that were injected with HPV-E5 developed tumors at a high frequency compared to transgenic mice that had mutations in the Epidermal Growth Factor Receptor (EGFR) [Genther Williams et al. 2005b]. This was later shown to occur through E5 coupling the Epidermal Growth Factor (EGF) which enhances its binding to Epidermal Growth Factor Receptor (EGFR) thereby, driving the cell into S phase [Genther Williams et al. 2005a]. HPV-E5 also functions by helping HPV infected cells evade the immune surveillance through down regulation of CD1d expression [Miura et al. 2010]. In addition to these functions, HPV E5 also promotes anchorage independent growth and it inhibits cell to cell communication [Suprynowicz et al. 2010]. Through these process HPV is able to maintain a low copy number of viral DNA in the basal cells.

When the daughter cells from the basal layer differentiate the HPV DNA in them undergoes replication through the expression of HPV E2 and E1 proteins [Graham. 2010]. These proteins are required for both replication of viral DNA and transcriptional regulation [Graham. 2010]. E2 and E1 bind cooperatively to the origin of replication. E1 is an ATPase helicase, and E2 is responsible for the recognition of origin of replication. This combination initiates the replication of DNA genome. E4 is also expressed at this stage and is necessary for binding the cytokeratin and blocking the cells in G2/M phase. It is the increased expression of E1and E2 that leads to a high viral DNA copy number and increased proliferation in stratified epithelial cells [Graham. 2010] causing microscopically observed hyperplasia of the cells.

Late gene proteins L1 and L2 are expressed in the highly differentiated epithelial cells. The L1 oncoprotein has been shown to be highly immunogenic [Christensen and Kreider. 1990] and is expressed in fully differentiated cells where there is less immune surveillance, so this could be another mechanism for the virus to evade the immune system. These late proteins form the viral capsid and are important for viral assembly and later for attachment during infection [Hindmarsh and Laimins. 2007]. The viral capsid has 72 capsomeres, each of which is made of L1 pentameres associated with L2 or more L2 proteins [Pittayakhajonwut and Angeletti. 2008]. The infectious mature virions are released as the fully differentiated epithelial cells peel off and can re-infect the neighboring cells or be transmitted to the next host.

Epidemiology of HPV

Persistent infection with the mucosal alpha Human Papillomavirus eventually leads to the development of either benign or malignant tumors [Coutlee et al. 1995, Hindmarsh and Laimins. 2007]. Studies have shown that high risk alpha-human papillomavirus (HR-HPV) is the etiological agent for more than 90% of cervical cancer, the second most common female cancer globally [Munoz et al. 2003, Walboomers et al. 1999, zur Hausen. 1996]. Penile cancer though of rare occurrence, has also been associated with HPV. HPV accounts for about 40% of penile cancers with HPV 16 having the highest prevalence [Guimaraes et al. 2011, Ng'andwe et al. 2007, Silva et al. 2011].HR-HPV is also associated with other anogenital and head and neck cancers[Arany et al. 1998, Graham. 2010, Miura et al. 2010, Smith et al. 2004].

HPV and HIV

Most individuals get exposed to HPV over their lifetime, but the majority are able to clear the virus through the induction of the immune system [Rowhani-Rahbar et al. 2007, Stanley. 2010]. Amongst individuals who are immunosuppressed, HPV infection persists because of the inability of the immune system to clear the virus [Arany et al. 1998, Wang et al. 2011]. The immune response in HIV infected individuals is compromised and this predisposes them to other infectious diseases including HPV. Several studies have reported increased prevalence of HPV infections among people infected with HIV compared to those who are HIV negative [Arany et al. 1998, Hawes et al. 2006, Minkoff et al. 2001] and the rates of HPV infection have also been shown to increase among

individuals with low CD4+ cells and high HIV viral load[Mbulawa et al. 2010, Wang et al. 2011].

In Sub Saharan Africa, the region with highest rates of HIV infection, increased rates of HPV infection and HPV associated diseases have also been reported. [Desruisseau et al. 2009, Louie et al. 2009, Ng'andwe et al. 2007]. In 2009 the incidence rates of Invasive Cervical Carcinoma in Sub Saharan Africa was 31.0 per 100,000 women, which was the highest in the world [Louie et al. 2009]. The mechanism of HIV and HPV association is not well understood. However, considering that cellular immunity is very important in viral clearance, it is probable that the loss in control of T cell distribution caused by HIV infection coupled with the down regulation of MHC I and CD1d by HPV helps to establish a persistent HPV infection. The other factor could be the route of transmission. Both HIV and HPV are sexually transmitted, so if proper preventive measures are not observed then one can get both infections at the same time.

HPV 16 Variants

The distribution of HPV genotypes differ by geographical regions, but HPV16 is the most prevalent high-risk genotype strongly associated with cervical cancer and accounts for about 50% of all cases globally [Kammer et al. 2000]. HPV16 has also been identified in anogenital cancers as well as head and neck carcinomas [Grulich et al. 2010, Muñoz et al. 2006]. HPV 16 is well studied because of its clinical significance and HPV 16 variants have been characterized. An HPV variant is defined as one that differs from other viruses of the same genotype by up to 2% in conserved regions of the genome, such as E1 or L1

ORFs, and by up to 5% in the LCR [Kammer et al. 2000]. HPV16 variants have varying degree of association with progression to cervical cancer, and this might explain why some people with HPV 16 progress to cancer faster than others. Differences in HPV 16 variants may be due to differences in transcriptional regulation by the virus or by biological properties of proteins encoded by HPV 16 variants. Studies and phylogenetic characterization of the conserved regions and LCR of HPV16 from cervical samples demonstrated that HPV16 variants can be divided into six distinct phylogenetic clusters distributed roughly by geographical regions; the European (E) group, two African (Af) groups, the Asian (As), Asian American (AA) and North American (NA) variants [Pande et al. 2008, Zuna et al. 2009, Zuna et al. 2009]. These variants also differ by oncogenic potential and epidemiologic studies indicate that non-European variants have increased oncogenic potential [Kammer et al. 2000].

L1 gene encodes the major capsid protein L1 and this protein is highly immunogenic [Murata et al. 2009]. Variations in the L1 region have been isolated from clinical samples and some of these have mutations that cause a conformational change within epitopes needed for binding the neutralizing antibodies [Pande et al. 2008]. These mutants are then able to resist clearance by antibodies mounted against the major HPV strain. Changes in the L1 region can also affect the ability of L1 to form Virus like particles.

CHAPTER II

INTRODUCTION

Introduction

Human Papillomaviruses are small non-enveloped, circular double stranded DNA molecules of about 7900bp in icosahedral capsids [Lowe et al. 2008]. The HPV genome encodes for eight proteins; early protein -E1, E2, E4, E5, E6 and E7 and late proteins L1 and L2. L1 forms the major capsid protein which is also important for the entry of the virus into the cell. HPVs form a diverse group with more than 100 types, at least 40 of these infect the genital area , with 15 types (HPV16, -18, -31, -33, -35, -39,-45, -51, -52, -56, -58, -59, -66, -68, and -73) classified as high risk HPV [Munoz et al. 2003]

Human Papillomavirus is strongly associated with development of cervical cancer. Most individuals infected with human Papillomavirus clear the infection without developing any recognizable symptoms [Rowhani-Rahbar et al. 2007, Stanley. 2010]. Progression to cancer is dependent on both host and viral factors. Viral type is one such viral factor. Infection with low risk HPV types can produce benign tumors or genital warts. However, persistent infection with high risk alpha human Papillomaviruses (HR-HPV) is the necessary cause for cervical cancer and other anogenital cancers [Chaturvedi. 2010].

The greatest antibody response in people infected with HPV are to the L1 protein [Murata et al. 2009], and the binding site for these type specific antibodies is a conformational epitope. In order to lessen the immune response, HPV produces L1 proteins in small quantities; later in the cycle and in terminally differentiated epithelial cells [Pittayakhajonwut and Angeletti. 2010]. The other possible mechanism that HPV can use to promote positive selection is to undergo mutation in the highly immunogenic

L1 protein, thus avoiding neutralization by the type specific antibodies that an individual could have developed either through vaccination or prior infection.

The immune status of the host plays an important role in the progression of the disease, and one study has shown an association between HIV negativity and HPV clearance [Banura et al. 2010]. While the specific mechanisms associated with increased susceptibility to HPV of HIV positive individuals are not fully understood, it is likely that the impaired cell mediated and humoral immunity present an advantage to progression of HPV infection toward a malignant state. HIV infection dramatically lowers or shifts populations of memory and effector T cells, effectively removing immune surveillance of newly forming neoplastic lesions. Report from one study has also shown that HIV has an influence in the expression of HPV genes [Arany et al. 1998].

Genotype specific HPV neutralizing antibodies are necessary for the prevention of infections and if an infection occurs, cell mediated immune response is needed to clear the infection but this immunity is impaired in HIV infected individuals [Stahl-Hennig et al. 2009]. Several studies have shown that there is high HPV prevalence among HIV infected people [Chaturvedi. 2010, Palefsky. 2009], and the association of HPV and HIV with progression of genital lesions to cancer has been well established [Firnhaber et al. 2010, Silverberg et al. 2002]. The higher prevalence of HPV related cancers amongst HIV infected individuals could also be due in part to availability of HAART which prolongs the life expectancy, thereby increasing risk for progression into cancer.

Sub-Saharan Africa has highest rates of HIV/AIDS and in 2008 it accounted for 67% of HIV infections worldwide [UNAIDS. 2009]. High rates of HPV infection have been reported from studies in this region especially among HIV positive individuals [Banura et al. 2010, Luchters et al. 2010, Moodley et al. 2009]. In Zambia, the University Teaching Hospital has reported HPV related cancer of the cervix as the leading cancer, representing 29% of all cancers seen in the hospital over the last 10 years [Bowa et al. 2009].

Persistent HR-HPV infections have also been associated with the development of penile cancer [Arany et al. 1998, Rombaldi et al. 2006], and like other HPV related cancers, the incidents of penile cancer are also higher among the HIV positive individuals. In the United States, HIV positive males have a 5-6 fold increased risk of developing penile cancer [Chaturvedi. 2010].

HR-HPV 16 type accounts for most cases of HPV related cervical cancers [Moodley et al. 2009] and HPV 16 variants based on sequence variation of the L1, L2, E6 and LCR have been defined. These HPV 16 variants can be divided into six different phylogenetic clusters: European (E) group, Asian (As), two African (Af) groups, Asian-American (AA), and North American (NA) group [Wheeler et al. 1997, Yamada et al. 1997]. HPV 16 isolates are defined as variants when the nucleotide sequence differs by up to 2% in conserved regions of the genome, such as E1 or L1 ORFs, and by up to 5% in the LCR [Kammer et al. 2000]. These HPV 16 variants show differences in their biological and biochemical properties. Epidemiologic studies have shown that non European HPV 16

variants have a higher oncogenic potential, with Asian-American being the most oncogenic [Kammer et al. 2000].

The purpose of this study is to look at the effect of the two variables; HIV and genital lesions on the HPV genotype concordance amongst heterosexual couples. We also wanted to analyze HPV 16 sequence variations in the L1 and LCR regions to determine the prevalence and novelty of HPV 16 variants. Understanding the effects of HIV and genital lesions on HPV genotype concordance and the knowledge of the circulating HPV 16 variants in the population will help in designing prevention strategies.

CHAPTER III

HUMAN PAPILLOMAVIRUS GENOTYPE CONCORDANCE WITHIN COUPLES

Study Participants

The study participants were recruited at the University Teaching Hospital Lusaka, Zambia (in collaboration with Dr. Kasonde Bowa). Individuals seen at the Urology clinic with any pathological penile lesion were recruited into the study. A penile lesion is defined as a macroscopically visible, acquired non-traumatic lesion on the glands, prepuce or penile shaft. For participation in the study, the index patient had to have a spouse or a regular female partner. A regular female partner is defined as someone with whom the index patient had regular sexual contact in the last six months. Three male participants, who were asymptomatic, were recruited into the study with their partners to serve as controls. The index patient and his partner were both recruited, and only included in the study after giving informed written consent.

Sample Collection

Two biopsy samples were collected from male partners; one sample was used for pathology evaluation and the other for HPV testing. The pathology results were classified as normal, benign/wart, malignant, ulcer or infection. The female participants had a cervico-vaginal lavage (CVL) and pap smear taken. The Pap smear was examined by a local consultant pathologist and the results were based on the Bethesda System for reporting cervical or vaginal cytological diagnoses [Apgar et al. 2003]. The CVL samples were treated with Qiagen DNeasy detergent solution, frozen at minus 20^0C, then shipped together with the penile lesion HPV samples to Nebraska Center for Virology at the University of Nebraska-Lincoln (UNL) for HPV testing.

HPV DNA detection and genotyping

DNA extraction was done using the Qiagen Blood and Tissue extraction kit (DNEASY). HPV detection was done using HPV consensus primers GP5+ (5'-TTTGTTACTGT GGTAGATACTAC-3') and GP6+ (5'-GAAAAATAAACTGTAAATCATATTC-3') [Ng'andwe et al. 2007] that amplify 150bp of L1 region. Beta actin amplification was done in all the samples as an internal quality control for the extracted DNA. Samples positive for HPV 16 were further amplified with HPV 16 specific primers for L1; L1 nt6155 (5'-GTCGGTACCCCATGTACCAATGTTGCAG3') and L1 nt7782 (5'CTACC CGGGGTGACATTTAGTTGGC CT-3') that amplify 1597 bp of the L1 region and part of the LCR. The reaction mixture of 50µl contained: 1 µl of the template DNA, 1.5mM Mglc2, 0.2mM deoxynucleotide triphosphate (dNTP), 0.5µM each of the primers and 1.25U Promega Go Taq® DNA Polymerase (Promega Corporation WI, USA). PCR conditions were set at: 94^0C for 5 minutes followed by 30 cycles of (95^0C for 30s, 62^0C for 1 min,72^0C for 90s) and 72^0C for 10 min. All amplifications included a positive control of HPV 16 DNA and a negative control of nuclease free water.

PCR products were separated either by 2%(for 150bp product and 0.7% (for 1597bp product) agarose gel using 1X Tris Acetate Ethylene-diamine-tetraacetic acid (TAE) buffer. At the end of electrophoresis, the gel was stained with ethidium bromide and visualized under ultraviolet light. The bands from positive samples were excised and isolated using the Qia quick gel extraction kit(Qiagen). The extracted DNA was cloned into pGEMT- Easy vector system (Promega Corporation WI, USA) by mixing 5ul ligase

with 50ng pGMET-Easy vector and 7.5ng DNA template. These were incubated at 4^0C overnight for maximum cloning efficiency.

The plasmids were transformed into DH5 alpha competent cells (100µl/plate) followed by incubation at 37^0C overnight in LBA plates containing 100µl (20 mg/ml) of isopropyl-beta-D-thiogalactopyranoside (IPTG) and 20µl (50 mg/ml) of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-GAL).This procedure allowed for selection of clones using blue white selection. Two white colonies were picked and cultured overnight at 37^0C in 5ml LB/Ampicillin(100ug/ml).The plasmid DNA was extracted using QIAprep Spin Miniprep Kit (QIAGEN Inc. CA, USA). To check for proper insert in the plasmid DNA, EcorI was used to digest 1µL of the DNA, and this was run on the agarose gel. The samples with inserts were sent for DNA sequencing and the BLAST alignment software (http://blast.ncbi.nlm.nih.gov/) was used to identify the genotypes and KALIGN software was used to align variants sequences. (http://www.ebi.ac.uk/)
Two independent PCR amplifications, cloning, and sequencing steps were performed to make certain that identified L1 sequence variants were not the result of Taq-derived mis-incorporations,

HIV testing

Blood samples were collected from participants who gave consent for HIV testing and diagnosis was done in a designated HIV laboratory following the Zambia Ministry of Health testing protocol. The technicians doing HIV testing were blinded from the HPV results and diagnosis and those doing HPV testing were blinded to the HIV status, until the testing was complete in both labs.

Results

Eighty eight individuals from ages 18 years to 66 years were initially recruited in the study, but fourteen did not meet the study inclusion criteria and were excluded. Reasons for exclusion were death of the partner, penile amputation and decline to participate(Figure 3.1). The average age for the remaining seventy four participants was 36(SD+/- 10) years for males while the average female age was 30(SD +/-8) years.

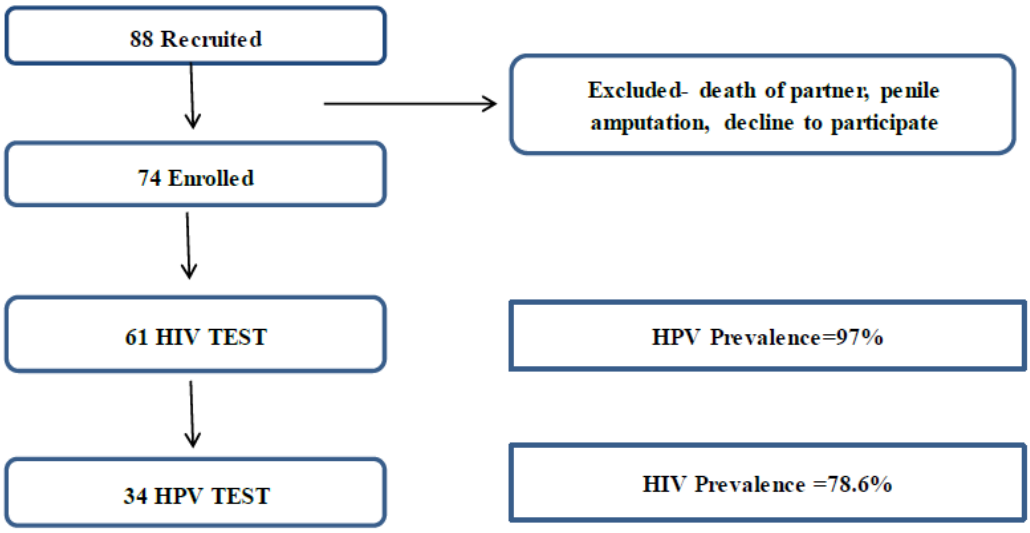
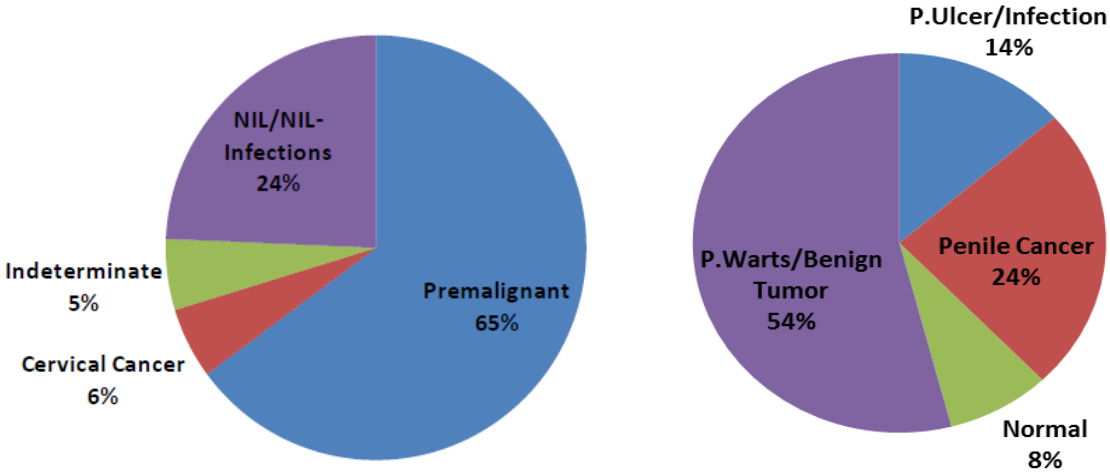


Figure 3.1 Summary of study Participants

Sixty one participants (82%) agreed to test for HIV. Among these, 48(78.6%) were HIV positive, and ten (21.4%) were HIV negative (Figure 3.1).

The penile pathology results were categorized into 4 groups; these were penile cancer, penile warts/benign tumor, penile ulcer/infection, and normal. The most common penile lesions were penile warts/benign tumor 20(54%) followed by penile cancer 9(24%).

Twenty four (65%) female partners had premalignant lesions and two (5.4%) had invasive cervical cancer. Nine (24.3%) females were negative for intraepithelial lesion(NIL) and of these two had cervical infections other than HPV. Two(5.4%) had an indeterminate result- loss of material or failure of processing(Figure 3.2).



a) Female lesions, n=37 **b)Male Lesions, n=37**

Figure 3.2 Distribution of genital Lesions. NIL-Negative for intraepitheial lesions.Indeterminate-loss of material or failure of processing.Premalignant category included cervical intraepithelial neoplasia(CIN) 1-3.

HIV and risk of cervical and penile lesions

HPV infection was closely associated with HIV positivity. Sixty eight (68%) percent of those who were HPV positive were also HIV positive compared to thirty (30%) percent being HIV negative. (Figure 3.3)

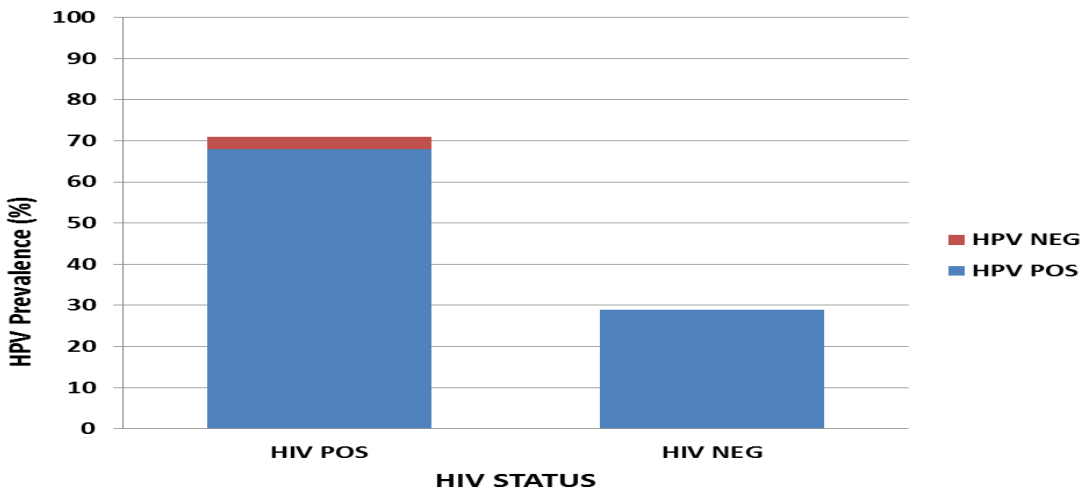


Figure 3.3 HPV prevalence by HIV status, N =34. HPV infection was closely associated with HIV positivity. Relative risk ratio 0.963 [0.89 1.036]

The HIV positive females were more likely to develop premalignant or malignant lesions, 69%, compared to HIV negative females at 3.8% (p=0.095).The risk of penile lesions amongst HIV positive individuals was 75% compared to 14.2%, hence overall HIV positive individuals were eight times more likely to develop cervical or penile lesions than negative individuals.(Table 3.1)

Table 3.1 Association of HIV status and genital lesions. HIV positive individuals are more likely to have cervical or penile lesions compared to those who are HIV negative.

	Number of lesions,(%)					
HIV status	**All**	**p-value**	**Females**	**p-value**	**males**	**p-value**
Positive	**39(72)**	**0.137**	**18(69)**	**0.095**	**21(75)**	**0.463**
Negative	**5(9)**		**1(3.8)**		**4(14.2)**	

HPV genotype distribution

Thirty four individuals (17 couples) were successfully tested for HPV. Out of these only one was found to be HPV negative. Genotypes identified in this group included HPVs -16,-35,-45,-58,-6,-11, and -83.HPV 16 was the most predominant, accounting for 18(53%) of all HPV types identified. (Figure 3.4)

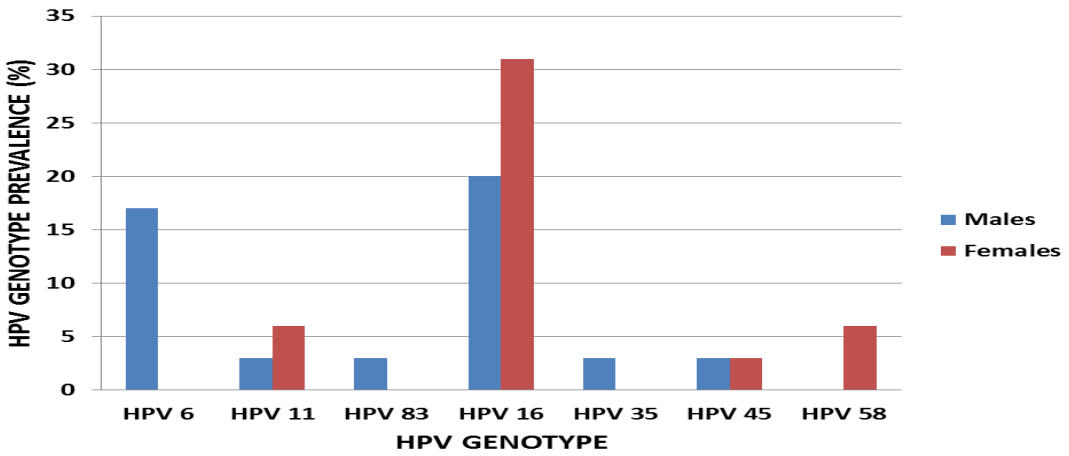


Figure 3.4 HPV genotype frequencies. The relative distribution of each genotype was determined. The x-axis represents the genotype and the y-axis represents the prevalence. N= 34.

Male samples genotyped included penile warts/benign tumor (9), penile ulcer/infections (2), penile cancer (3) and normal foreskins (3).These were all found to be HPV infected. Penile wart /benign tumor samples were shown to be associated with HPV 16, 6, and 11. The three asymptomatic male circumcision samples were all found to be HPV positive. These samples had HPV 16(2) and 83(1).Penile ulcer/infection samples were associated with HPV 16, 6 and 45. The only penile cancer sample genotyped was identified as HPV 6. The overall HPV distribution for this group of males was found to be predominated by HPV 16 followed by HPV 6 which were 20% and 17% respectively of male infections.

The less frequent HPV genotypes identified were 11, 83 and 45.Among the female partners who were HPV genotyped, one was identified to be HPV negative whose male partner was HPV positive and diagnosed with penile warts. Genotype frequencies observed in female partners were HPV 16 (31%),58 and 11 (6%) each and 45(3%). The 11 pre-malignant samples were associated with high risk HPV 16(9) and HPV 58 (2).

HPV genotype concordance within couples

Two HPV genotypes were found to be concordantly expressed between both male and female partners, HPV 16 and 11.A total of seven couples (43.8%) were found to have concordant HPV genotypes. Six couples had HPV 16 and one couple had HPV 11. Since the prevalence of HPV 16 was high in this cohort, and most of the couples that were concordant had HPV 16, we analyzed for HPV 16 variants concordance within couples. Out of the six couples that were concordant for HPV 16, only five shared the same HPV 16 variant. Among the six couples that had HPV sequence similarity concordant couples, 3 (50%) had concordant HIV positive results, one had concordant HIV negative results, and two couples had a discordant HIV results. Most of the male partners (67%) who had concordant HPV genotypes with their female partners had a penile lesion. Amongst the eight discordant couples, at least one partner in six couples was found to be harboring HPV 16. The other two couples had HPV 58 and 45. Some of the HPV 16 variants identified had mutations and we found that some of these mutations were the same among couples as shown in the example, Figure 3.4.

Table 3.3 Characterization of HPV genotype concordant couples. AF1- African 1; AF2-African 2; E- European.NIL-Negative for intraepitheial lesions.Cervical intraepithelial neoplasia(CIN) 1-3

Couple	Gender	Clinical	HIV	HPV	HPV 16 VARIANT
1	F	CIN2	Neg	16	AF1
	M	P.ULCER	Neg	16	AF1
2	F	CIN2	Pos	16	AF2
	M	P.WARTS	Pos	16	AF2
3	F	CIN2	Pos	16	AF1
	M	P.WARTS	Pos	16	AF2
4	F	NIL-Infection	Neg	16	E
	M	P.WARTS	Pos	16	E
5	F	CIN 1/2	Pos	16	AF1
	M	Normal	Pos	16	AF1
6	F	CIN 1	Pos	16	AF1
	M	Normal	Neg	16	AF1
7	F	NIL	Pos	11	n/a
	M	P.Warts	Pos	11	n/a

HPV L1 VARIANTS

There were 18 samples that were positive for HPV 16, and sequence analysis of the L1 region, showed that there was variation in HPV 16 amongst these participants. The most common HPV 16 variant, African group 1, made up 50% of the variants, the European group accounted for 33% while the African 2 variant made up 17% of the HPV 16 genotype. Some of these variants had mutations and T6313C was the most common mutation observed mainly in all AF1 group. We also observed T6495C in 17% of the variants. Some mutations were shared amongst couples indicating a long term evolution of the virus. Couple 4 had C6459T mutation while couple 6 had T6495C mutation.

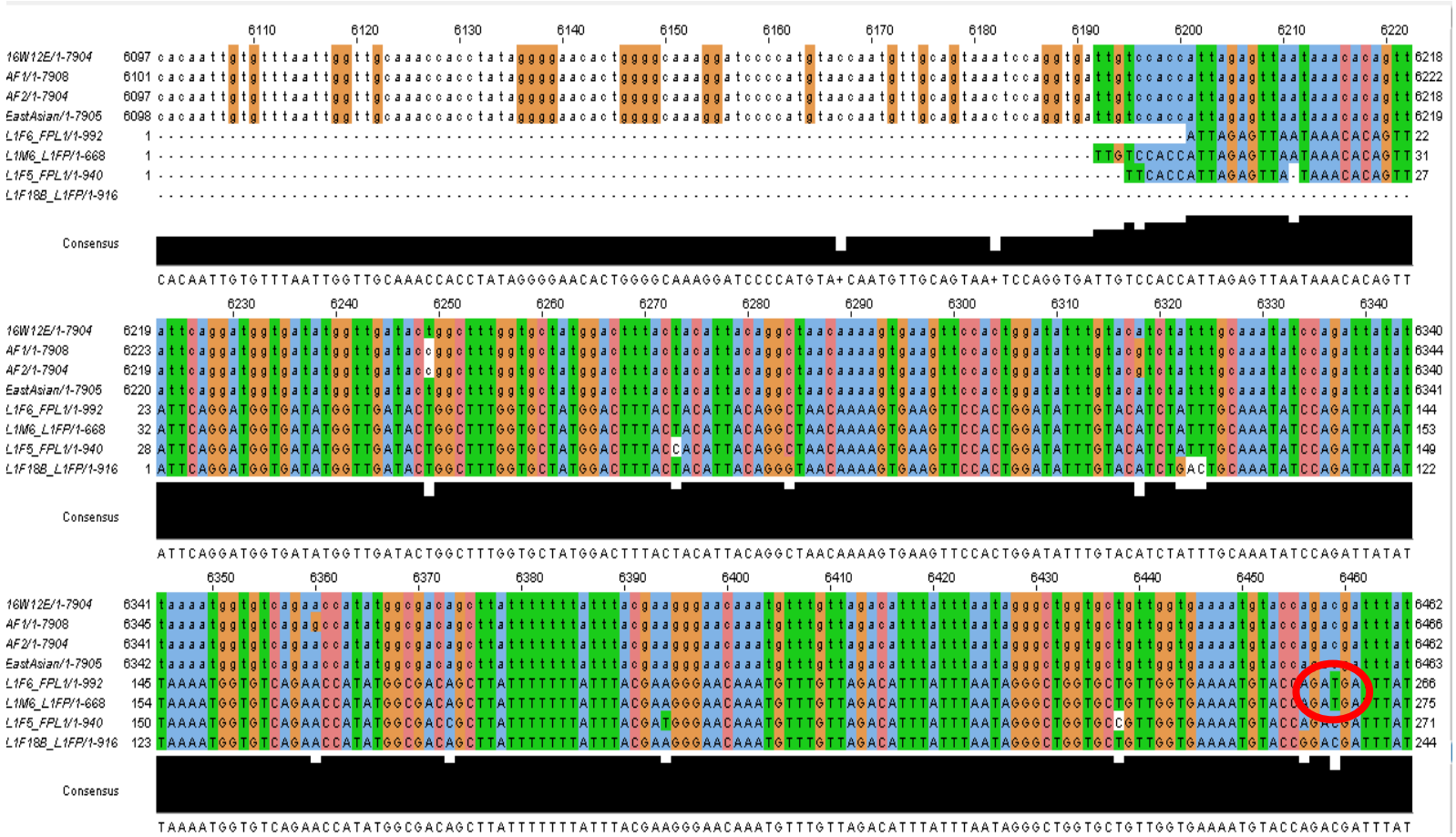


Figure 3.4 HPV 16 alignments. 16W12E, AF1, AF2 and East Asian are HPV 16 reference variants.L1F6 and L1M6 was an HPV 16 concordant couple. L1F5 and L1F18B were also HP16 positive

Discussion

In 2002, the number of new cervical cancer cases in developing countries was estimated at about 490,000 and 274,000 women died from invasive cancer of the uterine cervix induced by oncogenic genotypes of human Papillomavirus (HPV).In Zambia cancer of the cervix is the most common cancer with an incidence of 67.1 per 100,000 per year[Bowa et al. 2009, Parham et al. 2006].

Though the study sample is small some interesting discussion points are raised by the study. There was a high percentage of cervical lesions in the study cohort (70.4%), much higher than that observed in women with partners having penile lesions [Bleeker et al. 2002]. The study found a high proportion of premalignant and malignant lesions in female partners, which could be associated with the high prevalence of HIV and oncogenic high risk HPV subtypes, as shown by this study and other studies in Zambia [Bleeker et al. 2002, Sahasrabuddhe et al. 2007].The HIV infection suppresses cellular immunity which is useful in clearance of HPV infection. Our study has shown that HIV positive individuals are at least twice as likely to be infected with HPV, and the prevalence of High Risk (HR) types in HIV positive is twice as likely as the LR types. Previous studies have also shown that HPV infection is high in the general population and even higher in HIV infected populations[Bleeker et al. 2002, Sahasrabuddhe et al. 2007]**.**

The HIV prevalence was 78.6% which is much higher than the population prevalence of 14.3%. However, the high HPV co-infection rate could indicate both high risk behavior and high likelihood of HIV infection or simply due to a selection of a population with

much higher risk of infection, though a larger study would be needed to confirm this. Our study didn't establish the relationship between HIV status and HPV genotype concordance, and this could be due to small sample size, therefore more samples will need to be done. The presence of penile lesions seemed to increase the likelihood of a couple being HPV concordant. This study has also shown that heterosexual couples with active HPV infections can harbor identical or different HPV genotypes at any given time point. Females were found to harbor HR types at a greater rate than their male counterparts. These differences may be as a result of the site of specimen collection.HR HPVs would present a greater likely hood of establishing a persistent infection in the female genital tract,58.8% compared to 17.6% in males whereas male penile warts are likely to harbor LR HPV genotype,35.3% compared with 5.9% in females.

The difference in HPV genotype distribution could imply that despite repeated exposure to LR HPV genotype harbored in a male wart, the female genital tract clears the LR but maintains dominant HR infection. Other than clearance of the virus, the presence of discordant HPV genotypes amongst couples could also be due to multiple concurrent partners, but because not enough demographic data was collected, the next phase will address such questions.

HPV 16 was found to be the predominant type accounting for 52% of all HPV genotypes identified which is expected for an HIV endemic location .Previous studies have also reported high prevalence of oncogenic strains of HPV in Sub Saharan Africa where the rates of HIV are high [Baay et al. 2004, Firnhaber et al. 2010, Smith et al. 2007]**.** Interestingly, the control penile samples taken from asymptomatic adult male

circumcisions were found to be infected with HPV 16 and 83.Two of these controls were further found to have concordant genotypes with the female partners suggesting that in the absence of penile tissue abnormality it is likely couples will harbor the same dominant HPV genotype. The HPV 16 isolated from this group was found to have L1 variation. We observed AF1, European and AF2 variants in this population. Mutations at nt 6495, 6313 and 6459 were observed and this is indicative of the hyper-variability of L1 gene. The nt 6459 and 6495 are in the exterior loop of L1 protein, which is highly immunogenic. Mutations in this region may be important for viral fitness as they may cause conformational changes within epitopes needed for binding neutralizing antibodies.

CHAPTER IV

Human Papillomavirus 16 Variants from Zambian women with Normal Pap Smears

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Authors contributions

Yan Jun Lei – Sample testing and experiments

Kgomotso Makhaola- Manuscript preparation

Abstract

HPV 16 is the most prevalent high-risk viral genotype associated with cervical cancer. Six distinct phylogenetic clusters of HPVs have been identified and are distributed differently within five continents. HPV 16 DNA extracted from cervico-lavage samples from women with normal pap smears and the LCR regions were amplified in triplicate, cloned, sequenced, and analyzed. A total of 11 recovered HPV16 positive samples [Ng'andwe et al. (2007): BMC Infect Dis 7:77] were analyzed for sequence variation. The HPV16 LCR variants were assessed for promoter activity by use of the luciferase reporter gene. Six novel HPV 16 variants with nucleotide exchanges in the LCR region were identified. Five were classified as the HPV 16 variant European group, and one as the African group. Two of these variants had relatively lower promoter activity, 30% of that of the wild type strain. The decreased promoter activity of some HPV 16 variants decrease expression of viral oncogenes and may be linked with the development, phenotype and prognosis of the cervical lesions in women infected with these kinds of HPV 16 variants

Introduction

Epidemiological and molecular studies have demonstrated that high-risk alpha-human papillomavirus (HPV) is the etiological agent responsible for the majority of cervical cancers, the second most common female cancer globally [Walboomers et al., 1999; zur Hausen, 2000; Munoz et al., 2003]. So far, more than 120 different HPV types have been identified and characterized on the basis of DNA sequence analysis[de Villiers et al., 2004]. At least 40 HPV types infect the genital area, of which 15 types (HPV16, -18, -31,

-33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68, and-73) are classified as high-risk because of their oncogenic potential [Munoz et al., 2003]. HPV16 is the most prevalent high-risk type associated strongly with cervical cancer and accounts for about 50–60% of all cases of cervical cancer globally [Clifford et al., 2003].HPV16 has also been found in multiple cancers of the anus, vulva, vagina, and penis, as well as a subset of head and neck carcinomas [Koskinen et al., 2003; Ritchie et al., 2003].Although HPV genomes have been thought to be rather evolutionarily stable, nucleotide changes have been increasingly observed in clinical isolates. An HPV variant is defined as one that differs from other viruses of the same type by up to 2% in conserved regions of the genome, such as E1 or L1 ORFs, and by up to 5% in the LCR [Kammer et al., 2000]. HPV16 has been examined in great detail due to its medical importance, and numerous HPV16 variants have been identified. Studies and phylogenetic characterization of the LCR of HPV16 from worldwide cervical samples demonstrated that HPV16 variants can be divided into six distinct phylogenetic clusters distributed differently across the five continents: the European (E) group, two African (Af) groups, the Asian (As), Asian American (AA), and North American (NA) variants [Wheeler et al., 1997; Yamada et al., 1997]. Different HPV16 variants exhibit differences in their biological and biochemical properties. Epidemiologic studies indicate that non-European variants of HPV16 exhibit increased oncogenicity, specifically members of the Asian-American class AA [Kammer et al., 2000].

The early proteins of high-risk HPV, E6 and E7, play an important role in the carcinogenesis of human cells. They bind with and inhibit the function of the tumor suppressors p53 and pRb respectively [Dyson et al., 1989; Scheffner et al., 1990]. The

high-risk E6 and E7 protein also modify the expression activity of many cellular proteins in order to promote cell proliferation [Underbrink et al., 2008]. The expression level of HPV E7 is closely correlated with the transforming potential of the virus, and high levels of HPV E7 mRNA expression have been found in oral and cervical cancer biopsy specimens [Liu et al., 1995].Recently, studies have shown that HPV16 E5 also plays a critical role in mechanism of tumorigenesis [Hu et al., 2009]. The E1 and E2 proteins of HPV are required for both replication of viral DNA and transcriptional regulation [Longworth and Laimins, 2004]. The Long control region (LCR), an 800 bp long nonencoding region of HPV genome, contains an epithelial cell-specific enhancer, putative binding sites for cellular and viral transcription factors and the P97 promoter at its E6-proximal end. The transcription of HPV16 early genes initiates at the p97 early promoter and is controlled by a complex interaction of many cellular and viral factors that bind to the regulatory region in the LCR. Some of these factors include; YY1,NF1, SP1, AP1, TEF1, Oct-1, GRE, and NF-IL6 [Chan et al., 1990; Chong et al., 1991; Ishiji et al., 1992; Apt et al., 1994; May et al., 1994; O'Connor and Bernard,1995; Khare et al., 1997]. P97 activity plays the central role in the pathogenesis of HPV16. Exchange of nucleotides within transcription factor binding sites resulting in an increase in viral promoter activity and enhanced viral tumorigenesis, has been previously reported for HPV16 [Dong et al., 1994; Kammer et al., 2000; Tornesello et al., 2000]. One study reported that altered p97 activities are also primarily responsible for the strong differences in replication levels in HPV16 LCR variants [Hubert, 2005]. Although almost all cases of cervical cancer are attributable to HPV infection, infection alone is not sufficient to cause cancer. HPV DNA has often been found in normal cervical tissue

[Johnson et al., 1990; Woods et al., 1993]. The rate of HPV infection and cervical cancer is highest in the African continent. A previous study investigated the distribution of HPV genotypes in a population of Zambians with normal pap smears [Ng'andwe et al., 2007]. That study showed that HPV16 and HPV18 were the two most prevalent types, and each was present in 21.6% of Zambian samples, significantly higher than the average world rate [Ng'andwe et al., 2007]. In the present study, six novel HPV16 variants were identified by sequence changes in the LCR. HPV variants were isolated from vaginal lavage specimens from Zambian women with normal pap smears. To correlate LCR mutations with changes in p97 promoter activity, a luciferase reporter gene was cloned under the control of each LCR variant or the LCR of the HPV16 W12E isolate. The W12E HPV16 isolate falls within the European group and is widely used as a prototype for HPV16 studies since the full-length genome was cloned [Flores et al., 1999]. LCR variants that contained multiple mutations were systematically analyzed for the contribution of each point mutation to LCR promoter activity.

MATERIALS AND METHODS

Study Participants

This study reports an analysis of HPV16 variant distribution among HIV positive and negative women, sampled from a previously established cohort study [Ng'andwe et al., 2007]. All Human subjects protocols were approved by safety committees at the University of Zambia and UNL in accordance with the Helsinki Declaration. Patient participation was entirely voluntary and written consent was required for inclusion in

the study. Demographic details of the cohort participants from the metropolitan area of Lusaka were collected and disease histories as well as physical examinations were carried out to rule out any clinical symptoms or visible signs for these conditions. Pap smears were examined and classified according to the pap classification protocol; pap I (normal), pap II (inflammation), pap III (dysplasia), pap IV (carcinoma in situ), and Pap V (carcinoma), as described previously [Ng'andwe et al., 2007]. All of the participants chosen for this study had normal pap smears.

Sample Collection

Vaginal lavage samples and pap smears were collected from all patients. Vaginal lavage specimens, treated with DNeasy lysis buffer (Qiagen, Valencia, CA) were stored at -20^{0}C. All specimens were then shipped to the Nebraska Center for Virology at the University of Nebraska-Lincoln (UNL) for testing.

DNA Isolation

Total DNA was extracted from vaginal lavage samples using the DNeasy Tissue extraction kit (Qiagen). HPV16 infection in these samples was confirmed by PCR using GP5+/GP6+ and CPI/CPII primers followed by sequencing as detailed in the previous study [Ng'andwe et al., 2007].

PCR Amplification of the LCR

The LCR of HPV16 was amplified from the DNA samples by use of one pair of primers: P-LCR1: 5'-CCGAGCTCACGCAAAAAACGTAAGCTG-3' (nt7,133–7,150) with the SacI site underlined and P-LCR2: 5'-CCAAGCTTTCCTAGAACATTGCAGTTCTC-

3’ (nt 96–114) with the HindIII site underlined. These primers amplified a DNA fragment of 886 bp, corresponding to the complete LCR sequence of HPV16 (nt 7,133–114). The PCR amplification was performed in 50 ml of the reaction mixture containing

2 ml template DNA, 1.5 mM MgCl2, 10 mM dNTP,10 pmol of each primer and 5 U of Taq polymerase (Invitrogen, San Diego) at the cycle condition of denaturing at 948C for 50 sec, annealing at 588C for 50 sec, extension at 728C for 1 min, totally 30 cycles.

PCR products were analyzed on 2% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen), and then sequenced on both strands for the entire LCR of HPV16. To ensure that identified LCR sequence variants were not the result of Taq-derived mis-incorporations, three independent PCR amplifications, cloning, and sequencing steps were performed. The sequence data was compared with the corresponding fragment of HPV16 W12E isolate by use of BLAST alignment software online (http://blast.ncbi.nlm.nih.gov/).

Plasmid Constructions

The luciferase reporter vector under control of the LCR from HPV16 isolates (pGL3-LCR) were constructed by cloning the entire LCR from HPV16 variants, as well as the W12E isolate into the SacI and HindIII multiple cloning site of pGL3-basic plasmid

(Promega, MadisonQ3). The generated recombinant plasmids were: pGL3-LCR-101, pGL3-LCR-110, pGL3-LCR-902, pGL3-LCR-374, pGL3-LCR-536,pGL3-LCR-434, and pGL3-LCR-W12E. The recombinant plasmids to be used in transfection experiments were prepared using QIAfilter Plasmid Maxi Kit (Qiagen).The mutant constructs containing the individual point mutations that existed in the LCR of 374 and 110 HPV16

variants were created using the Quick Change II XL Site-Directed Mutagenesis kit (Stratagene, San DiegoQ4). The point mutations were introduced to LCR of HPV16 W12E isolate by PCR with various primers containing mutation at nt 7,405, nt7,416, nt 7,632, nt 7,742, nt 7,797 in variant 374, and nt 47, nt 7,405, nt 7,826 in variant 110. The parent plasmid was removed by digestion with DpnI, and further transformation was carried out according to the manufacturer's instructions. The mutant recombinant plasmids pGL3-374-7405 Mu, pGL3-374-7416 Mu, pGL3-374-7632 Mu, pGL3-374-7742 Mu, pGL3-374-7797 Mu, pGL3-110-47 Mu, pGL3-110-7826 Mu, and pGL3-374-7405 Mu were generated.

Cell Culture and Transient Transfection

HeLa cells and HaCat cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were seeded at a density of 2 $x10^5$ cells per well of six-well plates 1 day prior to transfection. Two micrograms plasmids of pGL3-LCR containing the distinct LCRs from the different HPV16 variants, mutated LCRs or W12E LCR prototype were transfected into monolayer cells together with 1 mg pCMV-b-galactosidase expression plasmid that served as an internal control to normalize for transfection efficiency. The luciferase activity was measured at 48 hr post-transfection with the Luciferase Assay system (Promega) according to the manufacturer's protocol. The relative light units were determined using a luminometer. The expression of b-galactosidase activity was determined using O-nitrophenyl-b-D-galactopyranoside (ONPG) as a colorimetric substrate. Relative luciferase activity measurements of the different plasmids were normalized against b-galactosidase activity. The averages were based on the mean of five independent experiments.

RESULTS

Identification of HPV16 Variants

A total of 11 HPV16 positive patient samples were recovered in a previous study [Ng'andwe et al., 2007]. DNA fragments containing the HPV16 LCR (nt 7,133–114) were amplified from HPV16 positive vaginal lavage specimens of Zambian women who had normal pap smears. We searched for genetic variation in the LCRs from the 11 HPV16 positive samples by DNA sequencing. Five wild-type (European) HPV16 LCRs and six novel HPV16 variants were recovered with nucleotide changes in the LCR sequence, not previously described (Table 4.1). Five new variants; 902,110, 374, 434, and 536 were classified within the European group and they exhibited 2, 4, 6, 1 and 3 intra-type variations, respectively, compared to HPV16 W12E isolates. The rate of mutation that accounts for 902, 110, 374, 434, and 536 variants in LCR appears to be above the intrinsic cellular mutation rate. Only one variant, 101, classified as African group, differed by two nucleotides, at nt 7,416 and nt 7,797, from the sequence of the Af1a B2 isolate published by Kammer et al. [2000]. The G to A transition at nt 7,797 was found in all variants except for variant 902. In contrast to nucleotide substitutions in the LCR of HPV variants described previously, several nucleotide insertions were found within LCR sequence of new HPV16 variants identified in this study. These data suggest that the diversity of HPV variants in natural infections has been underappreciated.

Two HPV16 Variants Isolated From Zambian Women Have Relatively Lower Promoter Activities

In order to assess the influence of nucleotide alterations in LCR on promoter activity, we utilized the recombinant luciferase reporter plasmids containing distinct LCR sequences from six isolated HPV16 variants, as well as the HPV16 W12E isolate, each cloned upstream of a luciferase reporter gene (pGL3). Constructs were transiently transfected into HeLa and HaCat cells. The luciferase assay revealed that there were no significant differences in promoter activity of LCRs from 902, 101, 434, and 536 variants compared to that of HPV16 W12E prototype isolate.

TABLE 4.1. Nucleotide Mutations Identified in the LCRs of the HPV16 Variants. Nucleotide changes are indicated within each variant. Insertions are indicated by (In) and deletions by (Del). Predicted transcription factor binding sites ''BS'' are shown in the right-most column.

Position (nt)	Variants							Binding Site
	W12E	902	101	110	374	434	536	
31	c		t					Sp1
47				(In) a			(In) a	E2
83	a		c					
7405				(Del) t	(In) c			
7416	a		t		(In) g			
7487	g		a					
7632					(In) a			AP1
7687	c		a					
7712	t		a					NF1
7742	t	a			(In) g			
7743		(In) c						NF-1
7744					(In) a			
7762	c		t					
7784	c		t					
7797	g		a	a	a	a	a	TEF-1
7826				(In) a				YY-1,TEF1
7832	g		t				(In) a	AP1
7874	c		a					

However, the expression of luciferase under control of LCR from variants 110 and 374 decreased to 30% and 26.6% of that from W12E isolate in HeLa cells, and decreased to

27% and 34% in HaCat cells (Fig. 4.1). These data suggest that variation in LCR-p97 promoter function are tolerable and potentially important for HPV fitness. Previous studies have found that mutations in the LCR that alter p97 promoter function often significantly affect gene expression as well as viral copy number [Hubert, 2005].

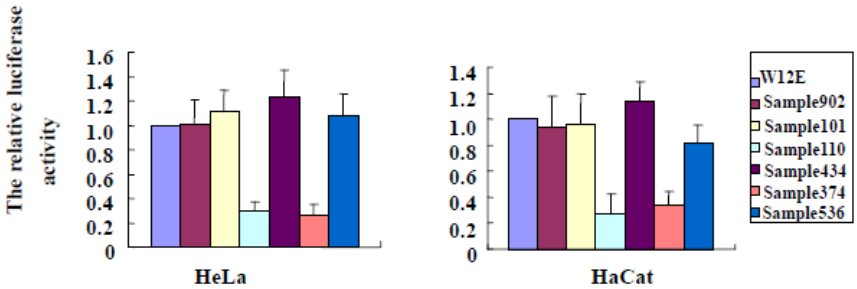

Fig. 4.1. The promoter activity under control of LCR from different variants as well as the W12E isolate is shown. HeLa cells and HaCat cells were transfected with 2 mg pGL3-LCR plasmids containing LCR of HPV16 W12E or different variants. The relative luciferase activity was averaged from five independent experiments in HeLa, HaCat cells and presented relative to that of W12E pGL3-LCR. The data are represented as the mean ±SD, indicated by error bars.

The Contribution of Different Point Mutations in LCR of HPV16 Variants 374 and 110 on Decreased Promoter Activities

To determine which of these point mutations in the LCR of HPV16 variants 374 and 110 decreased promoter activity of the virus, we introduced each different point mutation independently in LCR using the backbone of pGL3-LCR-W12E plasmid. The mutated plasmids were transfected into HeLa cells for analysis. The results showed that in the variant 374,the expression of luciferase under control of the mutated LCR with mutations at nt 7,405, nt 7,416, nt 7,632, and nt 7,797 were little compared to the plasmid with LCR from the W12E isolate. The plasmid containing the mutation at nt 7,742 was responsible

for a 70% reduction in the expression of luciferase (Fig. 4.2), corresponding to the expression level of luciferase under the control of LCR from variant 374 with all five point mutations. These results show that decreased promoter activity of variant 374 was primarily attributed to the point mutations at nt 7,742. In Electrophoretic mobility shift assay (EMSA) no difference was found in the binding of nuclear extracts prepared from HeLa cells with two probes (from nt 7,733 to nt 7,750) designed from HPV16 W12E and variant 374 (data not shown), yet the changes could still be affecting transcriptional activation. In the variant 110, the expression of luciferase under the control of the LCR with mutations at nt 7,405 decreased by about 87.5% and a mutation at nt 7,826 decreased to about 45%. The decrease in promoter activity in variant 110 maybe due to a synergistic effect between the two point mutations that existed in it. The mutation at nt 7,405 and nt 7,826 also did not induce an obvious difference in the binding affinity between nuclear extracts and DNA in EMSA results (data not shown).

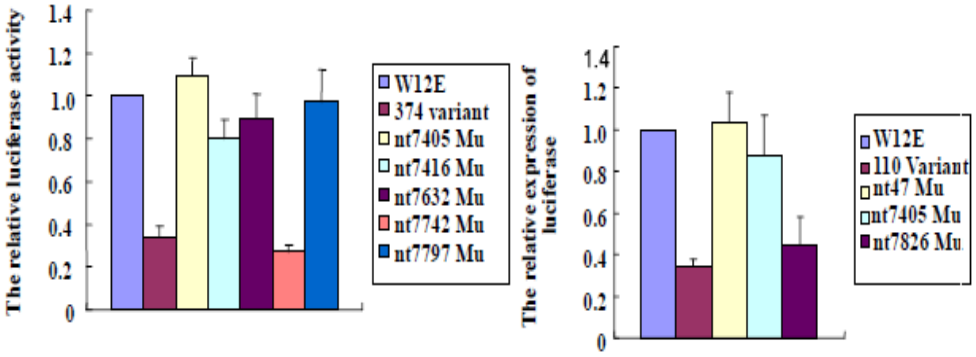

Fig. 4.2. The contribution of different point mutations found within the LCR of 374 or 110 variants on promoter activity compared to the W12E isolate. HeLa cells were transfected with 2 mg pGL3-LCR plasmids containing the LCR of HPV16 W12E or each combination of different point mutations from 374 or 110 variants respectively. The relative luciferase activity is the average of five independent experiments in HeLa cells and presented relative to that of W12E pGL3-LCR. Data are represented as the mean ±SD, indicated by the error bars.

DISCUSSION

In this study we identified novel HPV16 LCR sequence variants from cervico-vaginal lavage specimens isolated from Zambian women who had no evidence of HPV infection. Analysis of the LCR nucleotide sequences showed that five of six HPV16 variants were classified within the European group, while the other one, variant 101, was classified in the African group. Most studies on HPV16 variants have focused on isolates from clinical patients who already had cervical cancer or cervical squamous intraepithelial lesions (SIL). In contrast, the present study brings evidence of a significant number of LCR variant strains of HPV16 actively replicating in asymptomatic Zambian women. We focused on variants of the HPV16 LCR in this study since the p97 promoter has a crucial role in regulating transcription and replication of the virus. Although the direct biological consequence of variation in the LCR of HPV genomes on cervical carcinogenesis is not fully understood, several studies indicate that variants of HPV16 are associated with higher-risk of cervical neoplasias of various stages [Smits et al., 1994; Londesborough et al., 1996; Tornesello et al., 2000; Hildesheim et al., 2001; Burk et al., 2003; Hubert, 2005; Schlecht et al., 2005; Pande et al., 2008]. The mutations accrued in HPV genomes isolated from tumors are diverse in location and type, and thus the functional outcome is difficult to predictable. However, our current evidence of multiple mutations in the LCRs of actively replicating HPV16 genomes from women with normal pap-smears suggests that these differences are relevant to viral promoter activity and fitness. Relatively few studies have been done on HPV variants from women with normal pap-smears [Schmidt et al., 2001]. Non-European variants, particularly the Asian- American variants, tend to persist more frequently than E variants. In addition, non-E variants were more strongly

associated with both the prevalence and incidence of high-grade cervical intraepithelial neoplasia (CIN) lesions and cervical cancer than the E variants [Villa et al., 2000]. Kammer et al. [2000] reported that HPV16 Af1a and 2a variants exhibit p97 promoter activity comparable to the European reference clone. Thus, it may not be that surprising that HPV E and Af1a variants are found in women with normal Pap smears. It is possible that the distribution of variants is not only influenced by geographical region but also by severity of clinical phenotype. The results showed that six HPV16 LCR variants exhibited differing p97 promoter activity when linked upstream of the luciferase reporter gene. The 374 and 110 variants showed approximately a 70% decrease in p97 promoter activity, whereas other E group variants in this study and Af1a group, 101 variant were more or less as active as the HPV16 W12E promoter. This kind of large decrease in p97 promoter activity observed in variants 374 and 110, although rarely reported, might favor low-copy and long-term persistence of the virus, a reduced probability of cervical cancer. The importance of the transcription factor binding sites within LCR for transcription and expression of HPV16 early genes has been confirmed in previous studies [Hubert, 2005]. Single nucleotide changes can increase or decrease transcription factor affinity for binding sites or change their ability to interact with accessory proteins. Schmidt et al. [2001] reported that HPV16 LCR variants isolated from cancer cells contain nucleotide changes predominantly within or close to YY1 binding site. Variants obtained from asymptomatic carriers contained different single nucleotide changes, mainly within or close to binding sites of transcription factors such as AP-1, Oct-1, NF-1, TEF-1, TEF-2, SP1, viral E2, and also YY1 [Schmidt et al.,2001]. Not surprisingly, the results showed

that some of the nucleotide exchanges identified in LCR from the six different HPV16 variants were located near or within putative binding site for cellular or viral factors. The binding sites in the LCR variants 374 and 110 that resulted in a significant decrease in p97 promoter activity included AP1, NF-1, YY-1, TEF-1, and E2 binding sites (Fig. 4.3).

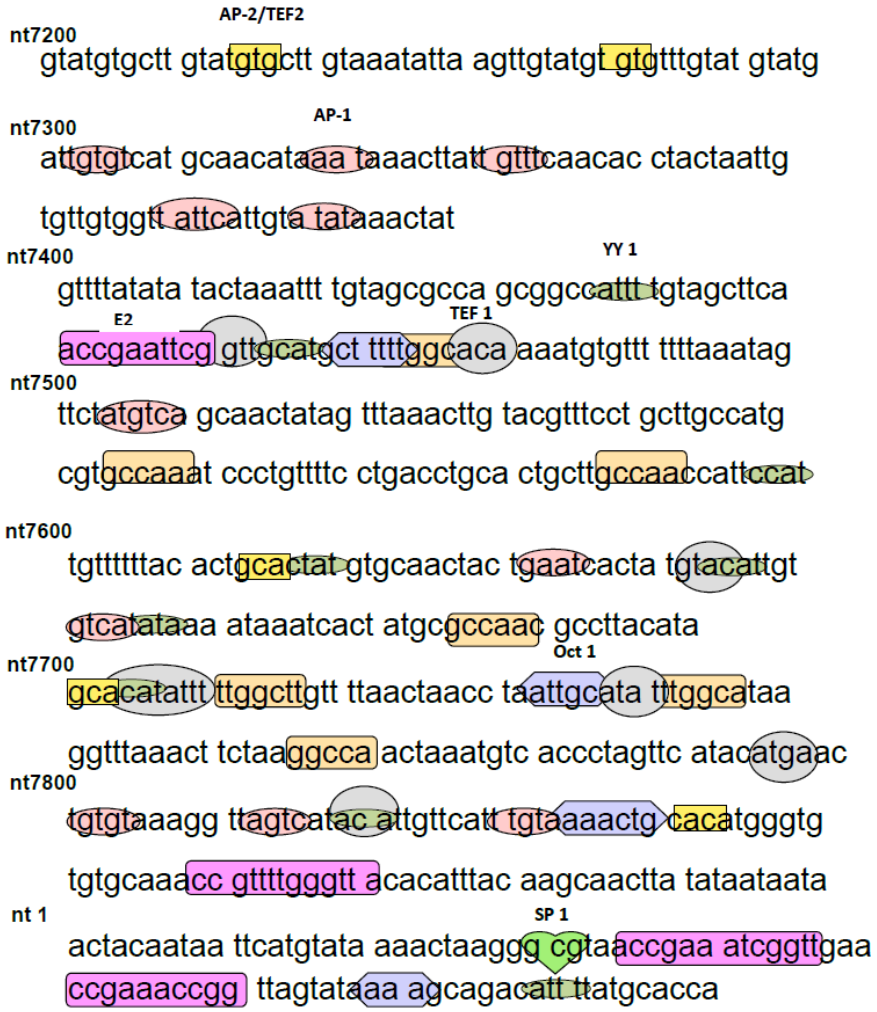

Fig. 4.3. Map of the putative transcription factor binding sites within LCR of HPV16. Each of the transcription factor binding sites are indicated; AP-2, AP-1, YY1, HPV16 E2, TEF1, NF-1, Sp1, and Oct-1.

By systematic analysis of point mutations, we identified the important nucleotide changes in variants 374 and 110 by their effects on p97 promoter activity. Only the LCR with nucleotide exchanges in the NF-1 binding site, a G insert at nt 7,742 and an A insert at nt 7,744, led to a decrease in p97 promoter activity to a level corresponding to that of 374 variant. These results indicate that the decreased p97 promoter activity of variant 374 was mainly attributed to affects on the NF-1 binding site near nt 7,742. As a ubiquitously expressed transcription factor, NF-1 binds to the regulatory elements within the LCR and plays a key role in the transcriptional stimulation of E6/E7 expression through the p97 promoter in HPV [Chong et al., 1991; Apt et al., 1994]. The nucleotide changes at NF-1 binding site that existed in LCR of variant 374 probably reduce the binding affinity between NF-1 and DNA sequence, thus partly decreasing the transcriptional activity through NF-1. However, the EMSA with HeLa cell nuclear extract was not able to detect differences in protein binding to oligonucleotides with or without mutations at nt 7,742 (as in variant 374 (data not shown)). In variant 110, a point mutation at nt 7,826 (corresponding to YY1 and TEF-1 binding sites) led to a major decrease in promoter activity. YY1 is considered a repressor for HPV16 transcription activity, and several YY1 binding sites have been reported in the HPV16 LCR. Single nucleotide mutations in YY1-binding sites that increase p97 activity three- to six fold have been identified in HPV 16 DNA from cervical carcinomas [Dong and Pfister, 1999]. In this study, we found a mutation at nt 7,826, within the YY1 binding site, which decreased p97 activity by about 50%. The YY1 binding site at nt 7,826 overlaps with the TEF-1 binding site. This may indicate competition for binding, displacement, or steric hindrance between the two proteins. We found it intriguing that there were a significant number of HPV16 variants

circulating in naturally occurring infections. The present results suggest that HPVs may exist in greater evolutionary diversity than previously appreciated. APOBEC3 cytosine deaminases have been described for their role in restricting infectivity of retroviruses by editing the single stranded DNA. Interestingly, a previous study has also shown that these deaminases are able to edit HPV 16 DNA [Vartanian et al., 2008]. Furthermore, APOBEC3 expression is known to be induced in the more terminally differentiated keratinocyte layers, where HPV genome amplification takes place. One of the variants we isolated, 101 did not show decreased promoter activity, but had point mutations typical of those induced by APOBEC 3a, 3c, and 3H [Vartanian et al., 2008]. This variant fell within the Africa group, which is thought to be less oncogenic compared to the European group. It is possible that at least some of this variation in HPV genomes is induced by mechanisms such as that described for APOBEC3 cytosine deaminases, but other mechanisms, such as polymerase driven insertions and deletions clearly also impact the pool of variants.

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CHAPTER V

GENERAL CONCLUSIONS

The purpose of this study was to look at the effect of the two variables; HIV and genital lesions on HPV concordance within heterosexual couples. We also wanted to analyze HPV 16 sequence variations in the L1 and LCR regions to determine the prevalence and novelty of HPV 16 variants. Understanding the effects of HIV and genital lesions on HPV concordance within couples and the knowledge of the circulating HPV 16 variants in the population will help in designing prevention strategies.

Though the study sample was small, we found evidence of HPV concordance within couples. The results also showed the mean age typical for men and women presenting with cervical and penile lesions. A previous study in Zambia has shown that there is a high prevalence of oncogenic HPV strains compared to elsewhere, and in this study most of the infections were attributed to HPV 16 and this means there is increased risk for premalignant and malignant lesions. This is indeed supported by a higher proportion of cervical lesions in this study cohort. Presence of genital lesions correlate with HPV concordance within partners, but we also found that individuals without lesions can still harbor same HPV types with their partners. However use of preventive strategies like male circumcision which has been shown to reduce penile lesions may be helpful in reducing this effect.

Several studies have found that HPV infection is highly associated with HIV positivity and we confirmed that with our results and a study before this one. In Zambia like other countries in Sub Saharan Africa, HPV infection is high in the general population and even higher in HIV infected individuals. The uptake of HIV testing in our cohort was 82 % much higher than the national average uptake of only about 10%.This was possibly due to the intense counseling and the fact that couples were already in the

clinical setting with a disease which may have motivated them to agree to HIV testing. The HIV prevalence was very high, however, the coinfection with HPV and possible ulcerative lesions could indicate both high risk behavior and potentiation of HIV infection or simply due to a selection of a population with much higher risk of infection. However due to small sample size we could not establish the correlation between HIV positivity and HPV concordance within partners.

The HPV genotype distribution differs by region, and HPV 16 has been found to be the most common of all genotypes globally with HPV 16 variants isolated from clinical samples. The defining character of HPV 16 variants is the difference in their oncogenicity and distribution by geographical region and it is possible that the distribution of HLA alleles in the population might influence this difference. We observed that HPV 16 variants are more prevalent in natural infections than previously perceived .We isolated L1 variants from the two African groups, and the European group as well as six novel HPV 16 variants with nucleotide exchanges in the LCR region. Five were classified as the HPV 16 variant European group, and one as the African group. Two of these variants had relatively lower promoter activity, 30% of that of the wild type strain. The decreased promoter activity of some HPV 16 variants decrease expression of viral oncogenes and may be linked with the development, phenotype and prognosis of the cervical lesions in women infected with these kinds of HPV 16 variants.

Our findings in this study provide additional information and give us more insight into the diversity of HPV 16 in the general population. We have also shown the use of variant analysis as a tool that can be used to trace genotype concordance within couples with more a higher accuracy. HPV variant data is important in developing vaccines and

other therapeutic approaches to control disease progression. Novel antiviral therapeutics that interfere with viral assembly and entry into cells would inhibit establishment of a persistent infection thereby preventing progression of tumorigenesis.

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