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**PLACENTAL HPV INFECTION IN HIV POSITIVE AND HIV NEGATIVE
ZAMBIAN WOMEN**

By

Chrispin Chisanga

A THESIS

**Presented to the Faculty of
The Graduate College at the University of Nebraska
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Under the Supervision of Professor Peter C. Angeletti

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PLACENTAL HPV INFECTION IN HIV NEGATIVE AND HIV POSITIVE ZAMBIAN WOMEN

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University of Nebraska, 2014

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Human papillomaviruses (HPVs) have been reported to infect epithelial trophoblastic cells of the placenta, induce cell death and even cause placental malfunction associated with spontaneous preterm delivery. To date, no study has been conducted to determine the role of HIV on HPV genotype distribution and pathogenesis in the placental compartment. This is despite the evidence that the human immunodeficiency virus (HIV) can decrease the cellular immune response and increase the incidence of malignant cancers in HPV patients. Therefore, in this study, we analyzed 200 genomic DNA (gDNA) samples extracted from paraffin embedded placental tissues of HIV positive and HIV negative Zambian women. The gDNA was PCR amplified using GP5+/GP6+ and CPI/CPII primers targeted to the L1 and E1 regions of the HPV genome, respectively. We found the overall prevalence of HPV to be 85.0%. The prevalence of HPV in the HIV+ tissues was 84(80.8%), while that of the HIV- tissues was 86(89.6%). This difference in HPV prevalence between the HIV+ and HIV- placental tissues was not significant ($p > 0.05$; $p = 0.112$). Direct sequencing of the PCR products revealed 3 HPV genotypes namely: HPV6, 16 and 90. We observed a significant difference ($p < 0.05$; $p = 0.0241$) in the high risk (HR) HPV16 incidence between the HIV+ and HIV- tissue with an odds ratio of 2.1. Because p16 is a surrogate marker for HR-HPV infection, we analyzed the placental tissue sections by p16 immunohistochemistry (IHC). The relative p16 signal per tissue area was significantly different ($p < 0.05$; $p = 0.0142$) between the HIV+/HPV16+ and

HIV-/HPV16+ groups. To confirm our L1 PCR findings, we also performed HPV16 L1 IHC and found that the relative L1signal per tissue area was significantly different ($p < 0.05$; $p = 0.0132$) between the HIV+/HPV16+ and HIV-/HPV16+ groups.

To the best of our knowledge, we are the first group to study HPV in the context of HIV within the placental compartment.

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CHAPTER I
LITERATURE REVIEW

Biology of Human Papillomaviruses

Human papillomaviruses (HPVs) are small, non-enveloped, double-stranded DNA (dsDNA) viruses (60) that infect squamous epithelial cells (10) and are responsible for benign warts of the hands, genitals and the larynx (25). To date, as many as 120 different types of HPVs have been identified, two-thirds of which infect cutaneous membranes and one-third of which infects mucosal membranes. Based on their ability to cause malignant carcinomas, the HPVs that target the mucosa can be categorized into the: high-risk type (HPV type 16, 18, 31) and the low-risk (HPV type 6, 11) type (27). The high-risk HPVs cause intraepithelial neoplastic lesions which can progress to cervical cancer, whereas the low-risk HPVs are responsible for genital warts (Condyloma acuminata) and express a rarity for progression to cancer (10).

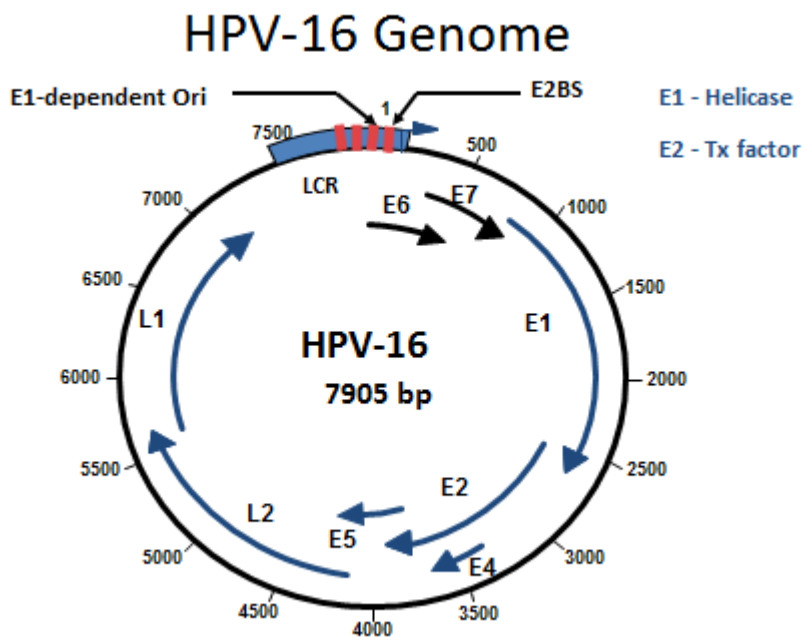


Figure 1.1 Organization of the HPV genome.

The HPV16 genome has 8 open reading frames (ORFs). Before productive replication occurs, the early proteins namely E1, E2, E6 and E7 are expressed. Expression of the late proteins (L1 and L2, including E1⁺ E4 and E5) occurs in the more terminally differentiated suprabasal cells.

The organization of the genes is the same in all human papillomaviruses (Figure 1.1). The HPV genome is approximately 8000 base pairs (bps) long and is composed of an early region in which non-structural genes (E1, E2, E4 and E5) are encoded; the late region which codes for structural genes (L1 and L2) and contains the long control region [LCR] (27). Each of the viral proteins plays a specific role; for example, E1 acts as a DNA helicase/ATPase (25) and in concert with E2, a transcriptional transactivator, induce viral DNA replication. Regulation of the cell cycle control and maintenance of the HPV viral genome is mediated by the E6 and E7 oncoproteins (19, 27). The E6 protein targets the tumor suppressor p53 for proteasomal degradation via the E3 ubiquitin ligase (E6-AP) complex (54, 73). Additionally, E6 activates the transcription of human telomerase reverse transcriptase (hTERT) which is a telomerase catalytic subunit (29, 73). The E7 oncoprotein binds and inactivates the retinoblastoma (Rb) protein, which acts as a brake in the cell cycle. The functional inactivation of Rb therefore prevents Rb from binding to the E2F transcription factors and consequently allows cells with inactivated Rb to proliferate uncontrollably (15, 37, 73). The biological role of the E4 protein in human papillomavirus infections has long been reported to be vague; however, recent studies have shown that the E4 N-terminal domain of both HPV16 and 18 enables the E4 protein to interact with cytokeratin. The C-terminal domain, however, allows E4 to induce cytoplasmic aggregate formation and to disintegrate the network structure of the cytokeratin intermediate filament (39). The E5 protein is a transmembrane protein that in the bovine papillomavirus (BPv) activates the platelet-derived growth factor β (PDGF- β) receptor tyrosine kinase by a mechanism that does not require a ligand. The BPV E5 protein induces receptor dimerization activation and, trans-phosphorylation and receptor-

associated recruitment of signaling proteins by forming a stable complex with the receptor. It is speculated that the E5 human papillomavirus protein could also affect the activity of PDGF- β and its signaling pathways in a similar fashion (12). The L1 protein is the major capsid protein which by itself can spontaneously assemble into virus-like particles (VLPs) in many expression systems of eukaryotes (17, 28). The role of the L2 protein is to bind DNA and mediate encapsidation (17, 82).

The HPV Life Cycle

The human papillomavirus life cycle is driven by the differentiation program of the infected keratinocytes. Infection of the basal layer cells with HPV initially occurs when there are minor abrasions or microtrauma in the epithelia (4). There is evidence to show that HPV entry into host cells occurs when virus like particles (VLPs) bind with specificity to the alpha 6 ($\alpha 6$) integrin subunit. The formed VLP- $\alpha 6$ complex containing either β_1 or β_4 integrin then serves as a receptor for the binding of the papillomavirus and therefore facilitates its entry into epithelial cells (17). The HPV life cycle is characterized by a genome maintenance phase, a proliferative phase, a genome amplification phase and a virus synthesis phase (14) in that order. The E1 and E2 proteins are thought to be expressed first, following infection and uncoating of the virus, in order to induce DNA replication (25) and establish a stable episomal viral DNA state (14, 74). The episomal viral genomes are tethered to the cellular factors via the E2 protein. This interaction results in the correct segregation of HPV genomes into daughter cells (36). During this phase, viral DNA replication occurs independently of the cell cycle and the viral copy number is amplified between 50 and 100 copies per cell. Furthermore, the expression of

high-risk HPV E6 and E7 oncogenes is regulated tightly and their respective transcripts cannot be detected in the proliferating epithelial compartment (61).

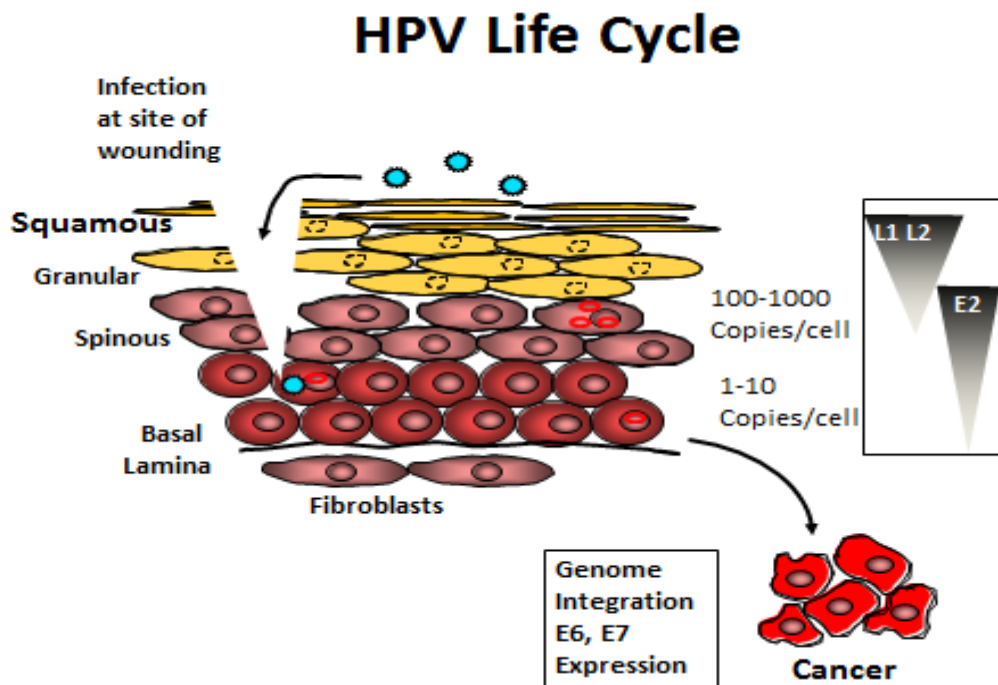


Figure 1.2 The human papillomavirus (HPV) life cycle.

Infection with HPV occurs when there is a micro-wound in the skin which acts as a conduit for the virus to the basal cells. The virus replicates episomally in basal cells at low copy number. The HPV viral life cycle is controlled by the host cell differentiation program. In the more terminally differentiated cells of the upper epithelial layers, productive life cycle occurs and this followed by the release of virion particles in the cornified keratinocytes

Genome maintenance is followed by a proliferative phase during which the number of basal cells harboring extrachromosomal viral DNA increases (14) to several thousands of copies per cell. This occurs when infected basal cells egress into the stratum spinosum, which is under active differentiation, and is accompanied by increased viral gene expression and viral DNA replication? Furthermore, the early genes (E6 and E7) are expressed abundantly and this is accompanied by the late gene expression from the late

promoter region (8, 61). In infections with the high-risk HPVs, E6 and E7 are expressed as oncogenes (14, 57). On the other hand, these proteins play no detectable oncogenic role in infections associated with the low-risk HPVs (34). The binding of the high-risk E6 transforming protein to the p53 tumor suppressor protein in concert with the E6AP cellular ubiquitin ligase (34, 55, 70) results in the targeting of p53 for proteasome degradation (61). The binding of the high-risk E7 oncogene to the unphosphorylated retinoblastoma tumor suppressor protein (Rb), phosphorylates the latter and releases the E2F transcription factor from the E2F-Rb complex. This allows E2F to bind cellular DNA and upregulate the expression of cell proliferation genes (69). The complementary role of E6 to E7 is thought to thwart apoptosis following the entry of the cell cycle into the S-phase (14).

During the late phase of the HPV lifecycle, the L1 and L2 proteins are assembled into icosahedral capsids. Virion assembly is accompanied by the release of mature viruses from the stratum corneum (34).

Biology of HPV and HIV Infection

Human papillomavirus (HPV) infections have been reported to be prevalent in human immunodeficiency virus (HIV) positive individuals (13). There is evidence to show that HIV-positive women have a high prevalence of HPV infections in the cervix (23, 42, 63) and a study conducted by Ahdieh and colleagues showed that HIV-positive women were 1.8, 2.1 and 2.7 times more likely to harbor high-, intermediate-, and low-risk HPV infections, respectively, than HIV-negative women. The persistence of HPV lesions was approximately twice greater in women with a CD4 cell count less than 200 cells/ μ l compared with greater than 500 cells/ μ l (1).

The number of sexual partners is one of the key risk factors for HPV acquisition. This is in agreement with findings that have suggested that individuals infected with HIV tend to have a higher prevalence of anogenital infections, (9, 42) with a lower CD4+ count being one of the most consistent risk factors for anogenital intraepithelial neoplasia. Thus, immune suppression due to HIV infection may play an important role in the development of high-grade intraepithelial neoplasia and eventual progression to cancer (42). The importance of cell-mediated immunity in the control of HPV infection has been evidenced by studies that have documented an increased prevalence and progression of HPV infections in the immunosuppressed (56, 59). Multiple recurrences of cervical HPV infections occur in HIV infected patients (20, 59). HIV may attenuate the systemic immune response against HPV via its effect on CD4+ cells and regulation of immune responses to different types of antigens. A low number of circulating HPV specific memory cells is thought to make the HPV-specific immunity vulnerable to the effects of HIV (42).

Biology of the Placenta

The placenta is an organ assembled from maternal and fetal cells and is involved in nourishing and protecting the fetus (81). Placentation is initially characterized by implantation of the blastocyst directly underneath the uterine epithelium followed by differentiation into embryonic and extra-embryonic tissues (5).

In humans, anchorage of the placenta in the uterine implantation site, known as the decidua, is mediated by invasive extravillous trophoblasts (EVT) which are involved in invading and restructuring maternal arteries to ensure that maternal blood flows into the intervillous space, bathing the fetally derived villous trees (81). The tertiary chorionic

villi consist of an outer layer of trophoblast covering mesoderm and blood vessels which connect proximally to the umbilical arteries and therefore separating maternal and fetal blood in a haemochorial pattern. The coordinated development of mature villous trees is vital for the growth and health of the fetus during the third-trimester of pregnancy. The villous trophoblast is a heterogeneous population of cells that make up the outer layer of the villi whose original columns of the cytotrophoblast are dispersed initially into a monolayer of cells that reside on a basal lamina. Finally, the proliferating stem cells give rise to the syncytiotrophoblast (5, 26) which plays an important role by providing resistance during pregnancy to a wide variety of pathogens, including cytomegalovirus [CMV] (18, 81), *Listeria monocytogens* (47, 81) and *Toxoplasma gondii* (48, 81). The blood-bathed surface of the syncytium cannot be breached by gastrointestinal pathogens such as *Listeria monocytogens* because it is void of E-cadherin, a host cell surface receptor that interacts with the virulent determinant internalin A protein (30, 81). Therefore, the lack of expression of E-cadherin and the absence of intercellular junctions on the syncytium is thought to be the main defensive mechanism by which adherence and internalization of *Listeria monocytogens* is thwarted (30, 47, 81). Additionally, invasion of host cells by *Listeria monocytogens* has been linked to abundant fused mitochondria (62, 81) which is unusually fragmented in the syncytium (68, 81). Thus, multiple unique biological properties make the syncytium an effective barrier to infection and the presence of a syncytium in placentas could be an evolutionary protective mechanism against blood-borne microbes and the transmigration of maternal leukocytes (11, 81).

Evidence of Placental HPV Infection

The high risk human papillomavirus type 16 has been shown to infect and productively replicate in 3A trophoblasts in tissue culture (77) and there is evidence to show that multiple HPV types 11, 18 and 31 can also replicate in these cells *in vivo* (78). Infection of embryonic trophoblast cells with HPV16 has been shown to result in spontaneous abortions (24). This occurs because HPV infection of extravillous trophoblasts induces cell death and may reduce placental invasion into the uterine wall (21). Prior work by Chan and colleagues showed that oocytes were capable of absorbing foreign DNA in the absence of sperm and that the zona pellucida had no barrier effect to the absorption of small DNA fragments by oocytes (6). This finding was supported by a subsequent study in which it was shown that mouse embryos at the blastocyst stage could passively and differentially take up exogenous human papillomavirus (HPV) DNA derived from the different HPV types 6b/11, 16 and 18 (7).

In vitro studies on mouse blastocysts after 24-hour exposure to HPV16 DNA have shown that DNA fragmentation occurs. This finding suggests that HPV type 16 may initiate apoptosis by disrupting DNA in the embryo (3) which might be one of the factors that lead to spontaneous preterm delivery (24).

Transplacental transmission of human papillomaviruses (HPVs) is well documented, with type-specific HPV concordance occurring between the mother, the placenta and the newborn or the mother and cord blood (50). A recent study showed that HPV DNA could be detected in 5% of neonates born to healthy women and that the HPV DNA could be associated with detection of HPV in mothers not only in the third, but also the first or

second trimester of pregnancy. There is a lot of controversy surrounding maternal-to-fetal transmission of HPV (31). This is because placental contamination with cervical cells from an infected birth canal cannot be ruled out. However, placental samples obtained from women undergoing trans-abdominal chorionic villous sampling have revealed the presence of HPV16 and HPV62 in a couple of placentas (71). Other studies have confirmed detecting HPV type 18 in both placental tissue samples and the cervix in pregnant women, with placental HPV infection being unrelated to the mode of child delivery (66). Further evidence by *In Situ* Hybridization has shown that HPV DNA can be localized in placental trophoblasts (52).

CHAPTER II
INTRODUCTION

Introduction

Human papillomaviruses are small, non-enveloped double-stranded DNA viruses that infect squamous epithelial cells via micro-abrasions which may occur on genitalia the (60). To date, approximately 200 different genotypes of HPVs have been identified (2, 61). Two-thirds of these infect cutaneous membranes and one-third infects mucosal membranes. Based on their ability to cause malignant carcinomas, the HPVs that target the mucosa can be categorized into the high-risk (HPV16,18,31) and the low-risk types (HPV6,11) (27). Infection with the low-risk HPVs usually result in benign epithelial warts, while infection with the High-risk HPVs leads to anogenital malignancies, including cervical cancer (58).

It has been shown previously that HPV16 and 31b can infect and replicate in 3A trophoblasts (33, 76).

Besides mediating nutrient and gas exchange between the fetus and mother, the fetal trophoblast cells are in direct contact with the maternal tissues and play a crucial role in placentation (32). Based on this intimate contact and communication between the maternal and fetal sides of the placenta, it is thought that infection with HPV16 may result in the death of placental trophoblasts, malfunction in the recognition capability of endometrial cells or malignancy. These changes may consequently disrupt the integrity of the trophoblast layer and cause spontaneous abortions or preterm delivery (32, 80).

The Human Immunodeficiency Virus (HIV) has been previously reported to decrease the cellular mediated immune response (42) and increase the incidence of HPV (56, 59). However, there is a dearth of information on the role of HIV on HPV genotype distribution and pathogenesis in the placental compartment. To this end, the present study

is aimed at understanding the extent to which HIV infection influences HPV infection and genotype distribution within the placental compartment. In regard to our main study objective, we determined the HPV genotypes harbored by placentas from HIV+ and HIV- women. We also determined the p16 and the HPV16 L1 major capsid protein levels in placental lesions from HIV-/HPV16+ and HIV+/HPV16+ samples. Finally, we determined sites of HPV infection by probing for HPV DNA in placental tissues.

Understanding the pathogenesis of HPV infection in the context of HIV infection within the placental compartment will provide insights into developing methods that can help prevent co-transmission of the two viruses from the mother to the child. This will be of particular importance in low income Sub-Saharan countries such as Zambia which are endemic to both HIV and HPV.

CHAPTER III
PLACENTAL HPV INFECTION IN HIV NEGATIVE AND HIV POSITIVE
ZAMBIAN WOMEN

Study Design

This was a Retrospective Cohort study in which the influence of HIV on HPV infection in the placenta was assessed.

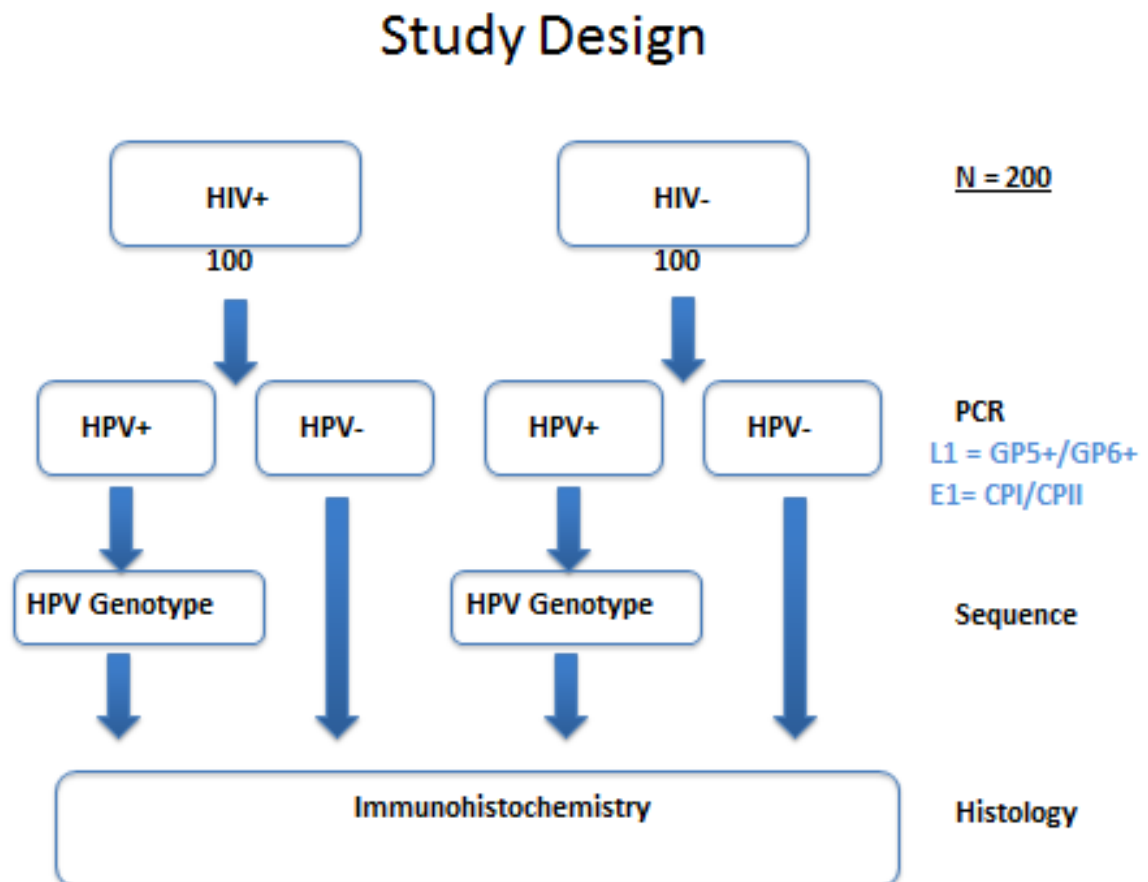


Figure 3.1 Placental HPV Infection Study Design

A cohort of 200 HIV+ and HIV- Paraffin Embedded Placental Tissues (PEPTs) was used in the study. Initially, genomic DNA was extracted and PCR amplified using GP5+/GP6+ and CPI/II primers. Beta (β)-actin primers were also used as controls. The expected 150bp PCR product was cloned into the pGEM-T Easy Vector System I followed by direct sequencing. Genotyping was achieved by aligning the sequences and blasting against the NCBI data base. Histological analysis of HPV16 in the tissues was done using immunohistochemistry (IHC)

Study Participants

The study participants were previously recruited at the University Teaching Hospital-Lusaka, Zambia. Informed consent of the patients was sought before the placental tissue samples were used in research study.

Sample Collection

A total of 200 Paraffin Embedded Placental Tissues (PEPTs) were obtained from Dr. Charles Wood's Laboratory Placental Tissue Bank. These tissues were obtained with informed consent from HIV negative and HIV positive Zambian women at the time of delivery. The samples were fixed in formalin and embedded in paraffin prior to being shipped to Nebraska Center for Virology (NCV) at University of Nebraska-Lincoln (UNL). To be included in the study, the placental tissue sample of the index patient had to be either HIV positive or HIV negative.

Genomic DNA Extraction

Genomic DNA was extracted from 200 paraffin embedded placental tissue samples using the Phenol-Chloroform extraction protocol as described by Pikor et al (43). Each of the paraffin embedded placental tissues was micro-sectioned and treated with 800 µl of xylene, to dissolve the paraffin wax from the tissues, followed by rehydration using a series of 800 µl ethanol (100%, 70% and 50%) washes. Tissue digestion was achieved by using 20 µl (20 mg/ml) of proteinase K, which was added in the morning and evening followed by incubation at 56⁰C in a heating block (Incublock™, Denville Scientific Inc.) for three consecutive days. This ensured that the tissue dissolved completely. The DNA

was cleaned up by the phenol-chloroform extraction method after which it was treated with 100 µg/ml of RNase A to remove any contaminating cellular RNA. Finally, the purified DNA was eluted in 50 µl nuclease free water and quantified by the Nano Drop Spectrophotometer (*ND-1000*).

Beta (β)-actin and HPV Amplification

The DNA extracted from placental tissue samples were amplified using regular PCR. Two redundant primers, (**GP5+**:5'-TTT GTT ACT GTG GTA GAT ACT AC-3' and **GP6+**:5'-GAA AAA TAA ATG TAA ATC ATA TTC-3') that amplify 150 bp of the L1 region (nt 6624-6746) of the HPV genome was used to detect HPV (40) in the samples. Another pair of redundant primers (**CPI**: 5'-TTA TCWTAT GCC CAY TGT ACC AT-3'-and **CPII**: 5'-ATG TTA ATW SAG CCW CCA AAA TT-3') which are targeted to the E1 region (nt 1777-1964) and amplify 188 bp of the HPV genome was also used to detect HPV in the samples. We also used β-Actin primers (**Forward Primer**: 5'-GCC ATG TAC GTT GCT ATC C-3' and **Reverse Primer**: 5'CCG CGC TCG GTG AGG ATC-3'). The use of these sets of primers on our samples and the simultaneous detection of HPV with both sets of primers provided a robust set of results for our analysis. The thermal cycler model, TECHNE, TC-412, was used for amplification. The parameters for denaturation, hybridization and extension were as follows: 94⁰C for 1 minute, followed by 30 cycles of 95⁰C for 30 seconds, 55⁰C for 1 minute, 72⁰C for 10 minutes and final hold at 4⁰C. The positive control constituted the HPV16 Plasmid DNA (pEF399), whereas the negative control was nuclease free water. To determine the presence or absence of HPV fragments and of Beta (β)-actin amplified from the oligonucleotides, 30µl of the PCR product from each sample was pre-mixed with 1.5 µl 6X loading dye

and separated by gel electrophoresis on 2% (w/v) agarose gel, in 1X TAE buffer. At the end of electrophoresis, the gel was stained with 0.3% ethidium bromide (0.1 mg/ μ l solution) for 30 minutes and visualization of the DNA fragments was performed under ultraviolet light.

Cloning of the PCR Products

Following PCR amplification of genomic DNA, the 150 bp amplicon was excised and purified from the 2% (w/v) agarose gel using the Qiaquick gel extraction kit (Qiagen) after which 1 μ l of the pcr product was cloned into the pGEMT-Easy Vector system I (Promega Corporation. WI, USA) and incubated at 4 $^{\circ}$ C overnight. The clones were then transformed into DH5-alpha (α) competent cells (100 μ l/plate), followed by incubation at 37 $^{\circ}$ C overnight on Luria Broth (LB) plates pretreated with 40 μ l (20mg/ml) of 5-bromo-4-chloro-3-indolyl-be-ta-D-galactopyranoside (XGAL) and 100 μ l (20mg/ml) of isopropyl-beta-D-thiogalactopyranoside (IPTG). Based on the Blue-white colony selection principle, two single white colonies from each plate were then isolated, cultured in 3ml LB containing 5 μ l of 50mg/ml Ampicillin and incubated at 37 $^{\circ}$ C for 12 hours in a shaking incubator. The plasmid DNA samples were then purified using the QIAprep Spin Mini-Prep Kit (Qiagen Inc. CA, USA) after which EcoR I Restriction Digestion of at least 1.5 μ g of Plasmid DNA was performed in a 20 μ l reaction volume to ensure that the plasmids had the correct 150 bp insert.

Sequencing of the HPV Clones

All the plasmid DNA samples containing the 150 bp insert were analyzed by Direct Sequencing using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing. The Direct Sequencing PCR Master Mix was performed in a 10ul reaction mixture containing 1.0 ul of 2pmol/ul GP5+ Forward Primer, 1.0 ul of 150ng/ul Plasmid DNA, 4.0ul Big Dye Mix and 4.0 ul of nuclease free water with the following PCR thermal profile reaction conditions: Hot start at 95⁰C for 5 m; 35 cycles of 95⁰C for 30s, annealing at 55⁰C for 15s, followed by extension at 60⁰C for 4 m and final hold at 4⁰C. This was followed by precipitation of the PCR product as recommended by the manufacturer. The HPV genotypes were determined by comparison with the NCBI GenBank database.

Immunohistochemistry (IHC) Staining

(a) Trophoblast Marker (HSD3B1) Immunohistochemistry

For this purpose, we raised a monoclonal antibody against hydroxyl-delta-5-steroid dehydrogenase (HSD3B1) as recommended by Mao et al (35). The slides containing the sectioned tissues were baked for an hour at 60⁰C in an incubator and allowed to cool for 30 minutes at room temperature before rehydrating with 5 minute incubations in Xylene 1 and 2, Absolute alcohol 1 and 2, 85% Alcohol and 75% Alcohol. Endogenous peroxidase in the tissues was blocked for 20 minutes with 2 mL 30% Hydrogen peroxide per 200 mL methanol, followed by rinsing in distilled water three times for three minutes. Next, the slides were treated in 0.02% Sodium citrate (v/v) and cooked for 20 minutes at 95⁰C to unmask the epitopes, after which they were cooled for 20 minutes at room temperature, while still immersed in the sodium citrate solution. This was followed by

rinsing in Phosphate Buffered Saline (PBS) for 5 minutes and rimming the tissue sections with a pap pen, carefully making sure that the slides did not dry. Blocking was achieved by incubating the slides in 150 μ L 10% Normal Goat Serum (10% NGS in PBS) for 30 minutes in a humidity chamber containing a little water. Next, the blocking solution was flicked off the slides and 150 μ L of the Monoclonal anti-HSD3B1 produced in mouse [SIGMA-ALDRICH] was added at a dilution of 1:2000. The primary antibody was, however, not added to the negative control slide. This was followed by an hour of incubation in a humidity chamber. Next, the slides were rinsed in PBS; three changes, three minutes each followed by addition of three drops of anti-mouse DAKO Envision + Horseradish Peroxidase (HRP) labeled Polymer (REF: K4001) and incubation in a humidity chamber for 30 minutes at room temperature. The slides were again rinsed in PBS; three changes; three minutes each. Using one slide, 200 μ L 2, 3-Diaminobenzidine (DAB) solution (1 drop of DAKO DAB+ Chromogen –REF: K3468 and 1 ml per 1mL of DAKO DAB+ substrate buffer- REF: K3468) was added and stain development was observed under the microscope, taking note of the time for optimal intensity. The rest of the slides were then developed using the same time that the DAB solution on the trial slide took to develop to the desired intensity. The slides were then washed in 200 mL of distilled water using two changes for five minutes each, followed by dipping them in undiluted hematoxylin for 20 seconds. The hematoxylin was washed off by letting the slides sit in running tap water for two minutes and then dipping them in ammonia water (500 μ L ammonia + 1000 mL of water) for 12 seconds, followed by dehydration sequentially as follows: 70% Alcohol; 85% Alcohol; 100% Alcohol; 100% Alcohol for five minutes each and two changes of Xylene for five minutes each.

Finally, cytooseal was used to coverslip the slides, leaving them overnight to dry before microscopic examination the following day.

(b) p16 Immunohistochemistry Staining

The same IHC protocol was used except that 150 μ L of the p16 primary antibody solution (1:20 dilution) was added, followed by 1 hour incubation in a humidity chamber containing a little water. The primary antibody used was p16 (JC8): sc-56330 purchased from SANTA CRUZ Biotechnology, Inc. This is a mouse monoclonal antibody raised against full length recombinant p16 of human origin. The negative control slide was stained in the absence of the primary antibody whereas the test samples were treated with both primary and secondary antibodies.

(c) p16 Quantification

For this purpose, two slides of each sample were used. Quantification of the p16 protein in both HIV positive and HIV negative tissue sections was performed using Image- Pro Version 9.0. The average p16 expression in each sample was normalized to the tissue area. We also quantified p16 expression in the HPV negative tissues. Statistical analysis to compare how the relative p16 expression varied across the groups was performed using the Kruskal- Wallis of GraphPad Prism 5. Further comparisons to determine differences, if any, in the relative p16 signal between the HIV-/HPV16- and HIV+/HPV16- as well as between the HIV-/HPV16+ and HIV+/HPV16+ groups were compared using the Mann-Whitney test.

(d) HPV16 L1 Immunohistochemistry Staining

Using the same IHC protocol we used an anti-V5 L1 monoclonal antibody (mAb) to probe for the HPV16 L1 protein in the placental trophoblasts. The mouse monoclonal

anti-V5L1 mAb is a type-specific neutralizing antibody which had been previously raised against human papillomavirus (HPV) type 16 L1 and was able to block more than 75% infectivity (67). In our study, a 1:250 dilution of the mouse monoclonal anti-L1 gave the best staining results for the L1 protein.

(e) HPV16 L1 Quantification

For this purpose, two slides of each sample were quantified and averaged. Quantification of the HPV16 L1 protein levels in both HIV negative and HIV positive tissue sections was performed using Image-Pro Version 9.0. The average HPV16 L1 protein expression in each sample was normalized to the tissue area. We also quantified HPV16 L1 expression in the HPV negative tissues to determine the baseline. As in the case of p16 quantification, statistical analysis to compare how the relative HPV16 L1 expression varied among groups was performed using the Kruskal-Wallis of GraphPad Prism 5. Further comparisons to determine differences, if any, in the relative HPV16 L1 signal between the HIV-/HPV16+ and HIV+/HPV16+ groups were compared using the Mann-Whitney test.

RESULTS

Patient Samples

Our study samples were composed of paraffin embedded placental tissues which were obtained from HIV positive and HIV negative women who had been admitted to the University Teaching Hospital (UTH), Lusaka, Zambia for delivery of their babies. These samples were then shipped to Nebraska Center for Virology (NCV) and stored in the Tissue Bank of Dr. Charles Wood, who kindly provided them for our HPV analyses. We chose to analyze these samples in order to determine if HPV infection was influenced by HIV. Very little is currently known about HPV infection in the placenta or the effect of HIV on those infections. Two-hundred samples, (100 HIV negative and 100 HIV positive) were analyzed.

Detection of HPV in Placental Tissues

HIV and HPV are endemic in Zambia. The prevalence rates for HPV as high as 97.2% have been previously reported among HIV positive Zambian women (51). Based on recent studies that suggest that HPV can infect epithelial linings of the placenta (53, 72), we decided to probe for the presence of HPV DNA in placental tissues of both HIV positive and HIV negative Zambian women. This goal of this study was to determine the effect of HIV upon HPV infection and pathogenesis in the placenta. For this purpose, we used a cohort of 200 placental tissue samples from which we extracted genomic DNA and PCR amplified HPV DNAs using GP5+/6+ and CPI/II primers. These redundant primers can amplify up to 40 different types of HPVs. The status of the cellular DNA in the samples was monitored by β -Actin PCR and any samples that did not test positive for

β -Actin PCR were excluded from the study. To this end, we excluded 4 samples from the 100 HIV negative samples based on their poor cellular DNA status. To make up for this decrease in the number of HIV negative samples, we added 4 more samples to the 100 HIV positive samples. Therefore, we finally had 96 HIV negative samples and 104 HIV positive genomic DNA samples for HPV analysis.

Figure 3.2 shows representative PCR results that were obtained by amplification of the L1 (nt 6624-6746) and E1 (nt 1777-1964) regions of the HPV genomes using GP5+/6+ and CPI/II primers, respectively. HPV DNAs were detectable in both HIV positive and HIV negative samples. A plasmid containing the entire HPV16 genome (pEF399) was used as a positive control.

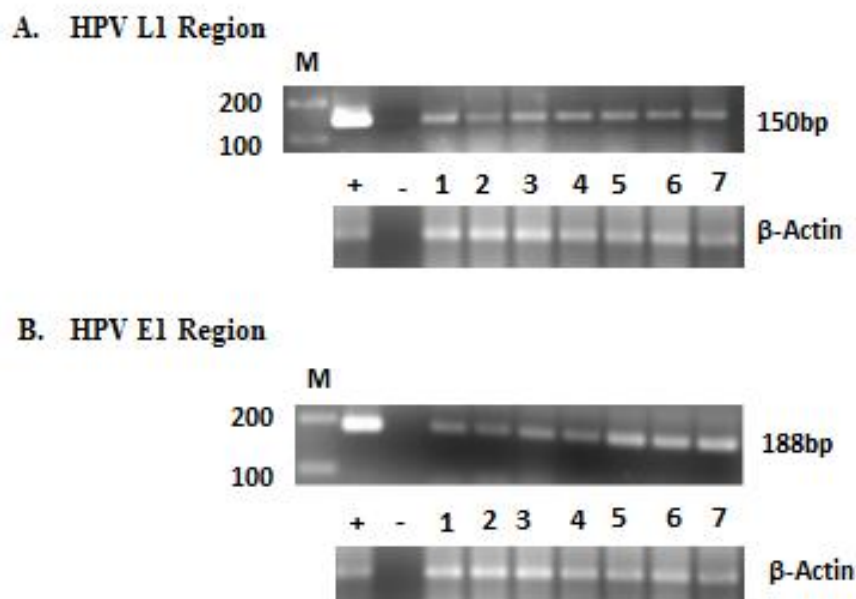


Figure 3.2 HPV PCR results obtained after amplification. (A) A 150bp PCR product from the HPV L1 region of HIV negative DNA using GP5+/6+ primers (B) 188bp of the HPV E1 region of HIV negative DNA using CPI/II primers. The cellular DNA status was assessed by β -Actin polymerase chain reaction. Nuclease free water was used as a negative (-) control, while pEF399 was used as a positive (+) control in GP5+/6+ PCR amplified samples, whereas gDNA for extracted from a B cell line was used as a positive control in β -Actin PCR amplified samples. The test samples were numbered as shown in Figures 3.2 (A) and (B). The HIV positive DNA samples were also PCR amplified in the same way (Results not shown). "M" is the marker.

Determination of HPV Prevalence

After detection of HPV in the DNA samples by PCR, the prevalence rate was determined. Figure 3.3 shows the prevalence of HPV in the study population. The overall HPV prevalence rate was 85.0%, with the HIV negative group accounting for 86(43.0%), and the HIV positive group accounting for 84(42.0%). Statistical analysis showed no significant difference (Fischer's Exact test: $p>0.5$; $p=0.112$) in the prevalence of HPV between these two groups.

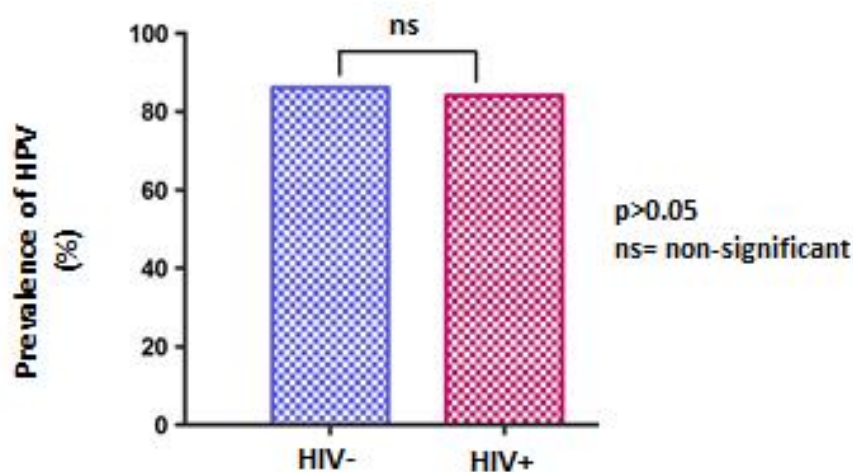


Figure 3.3 Prevalence of HPV in HIV- and HIV+ placental tissues

The prevalence of HPV in the HIV- placental tissues 86(89.6%) was higher than that of the HIV+ ones 84(80.8%). Comparison of the prevalence of HPV between the HIV+ and HIV- placental tissues using Fisher's Exact test showed no significant (ns) difference ($p>0.05$; $p=0.112$).

Having determined the prevalence of HPV in the placental samples (Figure 3.3), we sought to clone and sequence the PCR products in order to determine their HPV genotypes.

BLAST analysis of the sequences against the NCBI database revealed three types of HPVs in our cohort study. These were the Low-Risk (LR) HPV6, the High-Risk (HR)

HPV16 and the rarely reported HPV90. Eighty six (89.6%) HIV negative samples tested positive for HPV and 83(96.5%) of these were genotyped whereas the HPV genotype status of the remaining 3(3.5%) samples could not be determined. The HIV negative group harbored HPV90 which accounted for 4(5.0%) whereas the LR-HPV6 and HR-HPV16 accounted for 57(69.0%), and 22(26.0), respectively.

Of the 84(80.8%) HIV positive samples that tested positive for HPV, 83(98.8%) were genotyped while the HPV genotype status of 1(1.2%) sample could not be determined. The HPV positive group had the same HPV90 distribution 4(5.0%) as the HIV negative group. However, the HPV6 distribution in this group was 44(53%) and that of HPV16 was 35(42%). Comparison of the HPV16 distribution between the HIV+ (42%) and the HIV- (26%) groups using Fisher's exact test showed a statistically significant difference ($p < 0.05$; $p = 0.0241$) with a 2.1 odds ratio. We did not find a significant difference ($p > 0.05$; $p = 0.0864$) in the distribution of HPV6 between the two groups.

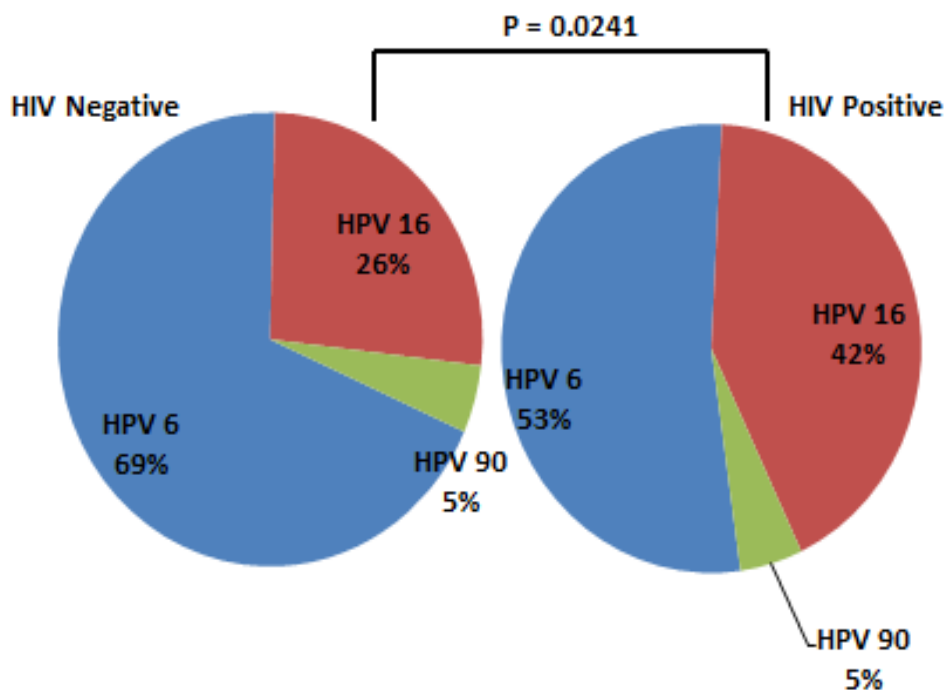


Figure 3.4 The distribution of HPV in HIV-negative and HIV-positive placental samples.

Three types of HPVs were isolated including the low risk (LR)-HPV6, high-risk (HR)-HPV16 and the rarely reported HPV90. (A) The pie-chart on the left side shows the distribution of HPV genotypes in HIV negative placental tissues and; (B) The pie-chart on the right side shows the distribution of HPV genotypes in HIV positive placental tissues. To determine the HPV genotypes, the PCR products were cloned into the pGMET-Easy Vector System I followed by EcoRI restriction digestion. The clones were subjected to Prism ABI Direct sequencing and the sequences were compared to the NCBI database for genotype identification. There was a significant difference ($p < 0.05$; $p = 0.0241$) in the distribution of HPV16 between the HIV-/HPV16+ and the HIV+/HPV16+ groups, with an odds ratio of 2.1. There was no significant difference ($p > 0.05$; $p = 0.0864$) in the distribution of HPV6 between the HIV-/HPV16+ and the HIV+/HPV16+ groups.

Socio-Demographic Characteristics of Women by HPV Status and Genotype Distribution

We wanted to know whether the socio-demographic characteristics of women (from whom the samples had been obtained) were related to the prevalence of particular HPV genotypes present in placental tissues.

Table 1 shows the socio-demographic characteristics of the women by HPV status and genotype distribution and HIV status. The data shows that overall; the most significant effects were in HIV-dependent effects on HPV genotype distribution. We found a significant difference (Fischer's Exact test: $p < 0.05$; $p = 0.0241$) in HPV16 genotype distribution between the HIV+ and HIV- women. There was no significant difference in HPV6 (Fischer's Exact test: $P > 0.05$; $p = 0.0864$) distribution between the two groups.

Furthermore comparison of age, years of education and household size between the HIV positive and HIV negative women did not reveal any significant difference (Fishers' Exact test: $p > 0.05$) in HPV genotype distribution.

Table 1: HIV Negative and HIV Positive Women

Table 1 shows the socio-demographic characteristics of HIV negative and HIV positive women by HPV status and genotype distribution. The HPV status and genotype distribution were compared with the marital status, age, and years of education. We also analyzed the effect of household size on HPV status and genotype distribution.

Table 1: Socio-demographic characteristics of women by HPV status and genotype distribution and HIV status												
	HPV-		HPV+		HPV16		HPV6		HPV90			
	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+	HIV-	HIV+		
Total	n= 10(10.8%)	n= 20(19.4%)	n=83(89.2%)	n=83(80.6%)	n=22(26.5%)	n=33(39.8%)	n=57(68.7%)	n=44(53.0%)	n=4(4.9%)	n=4(4.8%)		
Marital status												
Married	8(9.9%)	20(21.1%)	73(90.1%)	75(78.9%)	18(22.2%)	29(50.0%)	51(63.0%)	42(42.0%)	4(4.9%)	4(4.8%)		
Single	2(18.2%)	0(0.0%)	9(81.8%)	7(100.0%)	4(36.4%)	5(71.4%)	5(45.5%)	2(28.6%)	0(0.0%)	0(0.0%)		
Widowed	0(0.0%)	0(0.0%)	1(100%)	1(100%)	0(0.0%)	1(100%)	1(100%)	0(0.0%)	0(0.0%)	0(0.0%)		
Age (years)												
15-25	10(10.8%)	20(58.8%)	83(89.2%)	14(41.2%)	22(2.5%)	14(100%)	57(68.7%)	0(0.0%)	4(4.8%)	0(0.0%)		
26-36	0(0.0%)	0(0.0%)	0(0.0%)	62(100.0%)	0(0.0%)	19(30.6%)	0(0.0%)	43(69.4%)	0(0.0%)	0(0.0%)		
37-47	0(0.0%)	0(0.0%)	0(0.0%)	7(100.0%)	0(0.0%)	0(0.0%)	0(0.0%)	1(20.0%)	0(0.0%)	4(80.0%)		
Years of Education												
None	0(0.0%)	2(28.6%)	3(60.0%)	5(71.4%)	0(0.0%)	2(28.6%)	3(60.0%)	3(42.9%)	0(0.0%)	0(0.0%)		
1 to 7	4(8.3%)	8(19.5%)	44(91.7%)	34(82.9%)	13(27.1%)	21(51.2%)	30(62.5%)	13(31.7%)	1(2.1%)	0(0.0%)		
8 to 12	4(11.1%)	11(33.3%)	32(88.9%)	33(75.0%)	6(16.7%)	6(13.6%)	24(66.7%)	25(56.8%)	2(5.6%)	2(4.5%)		
>12	0(0.0%)	1(8.3%)	6(100.0%)	11(91.7%)	2(33.3%)	6(50.0%)	3(50.0%)	3(250%)	1(16.7%)	2(16.7%)		
Household Size												
1 to 3	5(11.4%)	7(18.9%)	39(88.6%)	30(81.8%)	15(34.1%)	14(37.8%)	21(47.7%)	15(40.5%)	3(6.8%)	1(2.7%)		
4 to 6	2(6.9%)	7(16.7%)	27(93.1%)	35(28.3%)	6(20.7%)	12(28.6%)	21(72.4%)	23(54.8%)	0(0.0%)	0(0.0%)		
7 to 9	2(18.2%)	3(17.6%)	9(81.8%)	14(82.4%)	0(0.0%)	5(35.7%)	9(81.8%)	6(35.3%)	0(0.0%)	3(17.6%)		
10 to 12	1(12.5%)	3(42.9%)	7(87.5%)	4(57.1%)	1(12.5%)	4(57.1%)	6(75.0%)	0(0.0%)	0(0.0%)	0(0.0%)		

Comparison of Resident HPV Genotypes in the Vaginal and Placental Compartments

Table 2 shows a comparison of the HPV genotypes resident in the vaginal and placental compartments of individual patients. The vaginal HPV study was previously conducted in our laboratory using lavage samples from same patients by the same PCR and genotyping methods as used for the present study. Twenty different types of HPVs were recovered from the vagina whereas only 3 HPV genotypes were recovered from the placenta.

Table 2: HPV Distribution in the Vagina and Placental Compartments

<u>Sample ID</u>	<u>HIV Status</u>	<u>Compartment</u>	
		<u>Vagina</u>	<u>Placenta</u>
79	-	None	6
112	-	None	90
132	-	None	None
919	-	6	6
78	-	None	6
186	-	81/62	6
1542	-	None	None
1543	-	None	None
107	+	45	6
126	+	None	6
64	+	None	16
81	+	16	None
133	+	51	None
184	+	83	None
984	+	53/6	None

Comparison of the vaginal lavage HPV types to those that we discovered in the placental compartment showed that the HPV genotypes resident in the two compartments were different, except for sample 919 which had a concordance of HPV6 in both compartments. We also noted that the vaginal compartment had mixed HPV infections while the placental compartment had none. Furthermore, we noted that infections were often exclusive to a single compartment (10/15 samples). The results of the analysis of HPV genotypes in the vaginal versus the placental compartments suggest that tissue compartment specific distributions of HPVs exist within individuals.

Trophoblast Marker (HSD3B1) Immunohistochemistry Staining

To identify epithelial syncytiotrophoblast cells, we used antibody to hydroxyl-delta-5-steroid dehydrogenase (HSD3B1) which exclusively expresses in terminally differentiated layer of trophoblasts cells. This marker has been shown to stain syncytiotrophoblasts in placental tissues with high specificity and sensitivity (35).

Figure 3.5A is a negative control that was stained in the absence of the primary antibody, while Figure 3.5B is the positive control tissue stained with the primary antibody. While no staining was observed in the negative control slide, the Trophoblast marker specifically stained the syncytiotrophoblastic cells on the outer surface of trophoblasts. The cytotrophoblastic cells were negative for HSD3B1 staining.

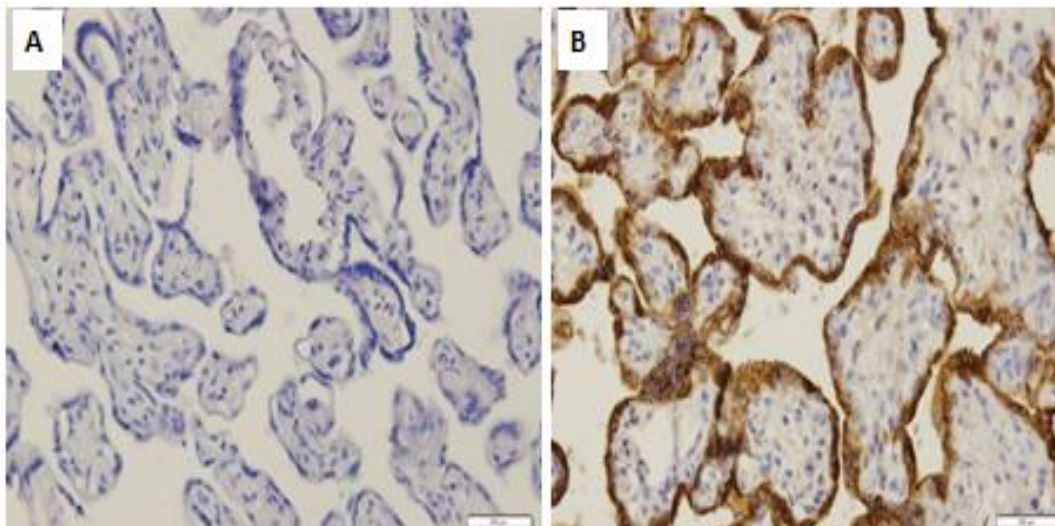


Figure 3.5 Immunoreactivity of HSD3B1 in fetal placenta. (A) HSD3B1 immunoreactivity in the presence of secondary antibody without primary antibody. (B) HSD3B1 immunoreactivity was detected in syncytiotrophoblasts. HSD3B1 was expressed as a brown pigment and characteristically stained trophoblastic columns. The cytotrophoblastic cells were negative for HSD3B1 immunoreactivity.

Detection of p16 Protein

The cyclin dependent kinase inhibitor (p16) is a cellular protein whose expression is elevated in infections with the high risk HPVs, such as HPV16. This occurs because the high risk HPV E7 oncogene binds the E2F-pRb complex and causes the release of the E2F transcription factors. This facilitates the binding of E7 to pRb which causes an up-regulation of p16. Therefore, p16 is a biomarker for the high risk HPVs (38, 46, 65) and to this end, we sought to determine the expression of p16 protein in HIV-/HPV16-, HIV+/HPV16-, HIV-/HPV16+ and HIV+/HPV16+ placental tissues. The expression of p16 protein was observed in all the four groups of placental tissues (Figure 3.5). The control conditions without primary p16 antibody showed no background staining of the placental tissue (Figure 3.5 B).

The p16 staining was observed both in cytotrophoblastic and syncytiotrophoblastic cells of the tissues whose genomic DNA had previously tested positive for HPV16 PCR. The expression of p16 in the HIV+/HPV16+ placental tissue (Figure 3.6E) appeared to be more disseminated and intense than in the HIV-/HPV16+ tissue (Figure 3.6D). The p16 staining was also observed in HPV negative tissues (Figure 3.6C). In all the tissue sections examined, p16 staining was both nuclear and cytoplasmic.

Since p16 expression is upregulated during high-risk HPV infections (38, 46, 65), we sought to determine whether this expression was augmented by the presence of HIV (Figure 3.6F). For this purpose, we used Image-Pro Premier Offline 9.0 to quantify the expression of p16 in HIV+/HPV16+ and HIV-/HPV16+ tissues. The expression of p16 signal was normalized to the tissue area and the relative p16 expression levels in the four groups were compared using Kruskal Wallis test. The medians for the relative p16 signal across the four groups varied significantly ($p < 0.05$; $p = 0.0367$). Comparison of the relative p16 signal between the HIV+/HPV16+ and the HIV-/HPV16+ placenta tissues showed a statistically significant difference ($p < 0.05$; $p = 0.0142$). However, there was no significant difference in relative p16 signal between the HIV-/HPV16- and the HIV+/HPV16- placental tissues ($p > 0.05$; $p = 0.4836$).

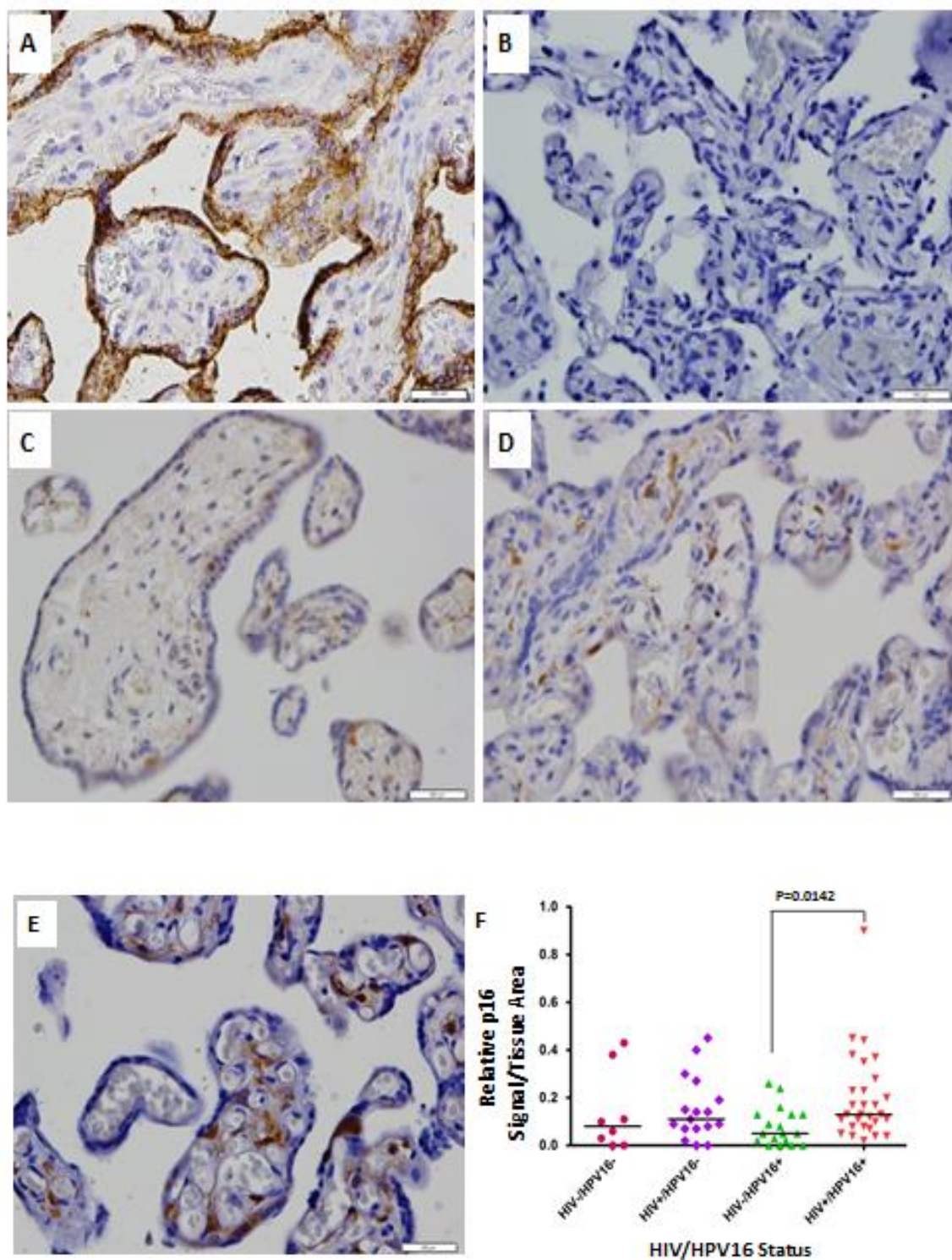


Figure 3.6 p16 immunohistochemistry staining. The p16 immunoreactivity was determined in the sectioned tissues (A) HSD3B1 trophoblast marker (B) Negative control in the absence of primary antibody. (C) p16 staining in an HIV-/HPV16- tissue. (D) p16 staining in an HIV-/HPV16+ tissue (E) p16 staining in an HIV+/HPV16+ tissue (F) Quantification of p16 protein in HIV-/HPV16+ and HIV+/HPV16+ sectioned placental tissues. Image Pro-Premier offline 9.0 was used to determine the p16 levels in HIV-/HPV16+ and HIV+/HPV16+ sectioned placental tissues. The p16 in levels both groups were determined in duplicate (Results not shown) and the average p16 values, normalized to the tissue areas, were compared using Kruskal-Wallis test. The bars represent the median p16 signal. The median p16 signal varied significantly ($p < 0.05$; $p = 0.0367$) across all the groups. Using the Mann-Whitney test, we found that there was a significant difference in the p16 signal ($p < 0.05$; $p = 0.0142$) between the HIV-/HPV16+ and HIV+/HPV16+ groups. We observed an HIV dependent effect on the expression of p16 in placental trophoblasts.

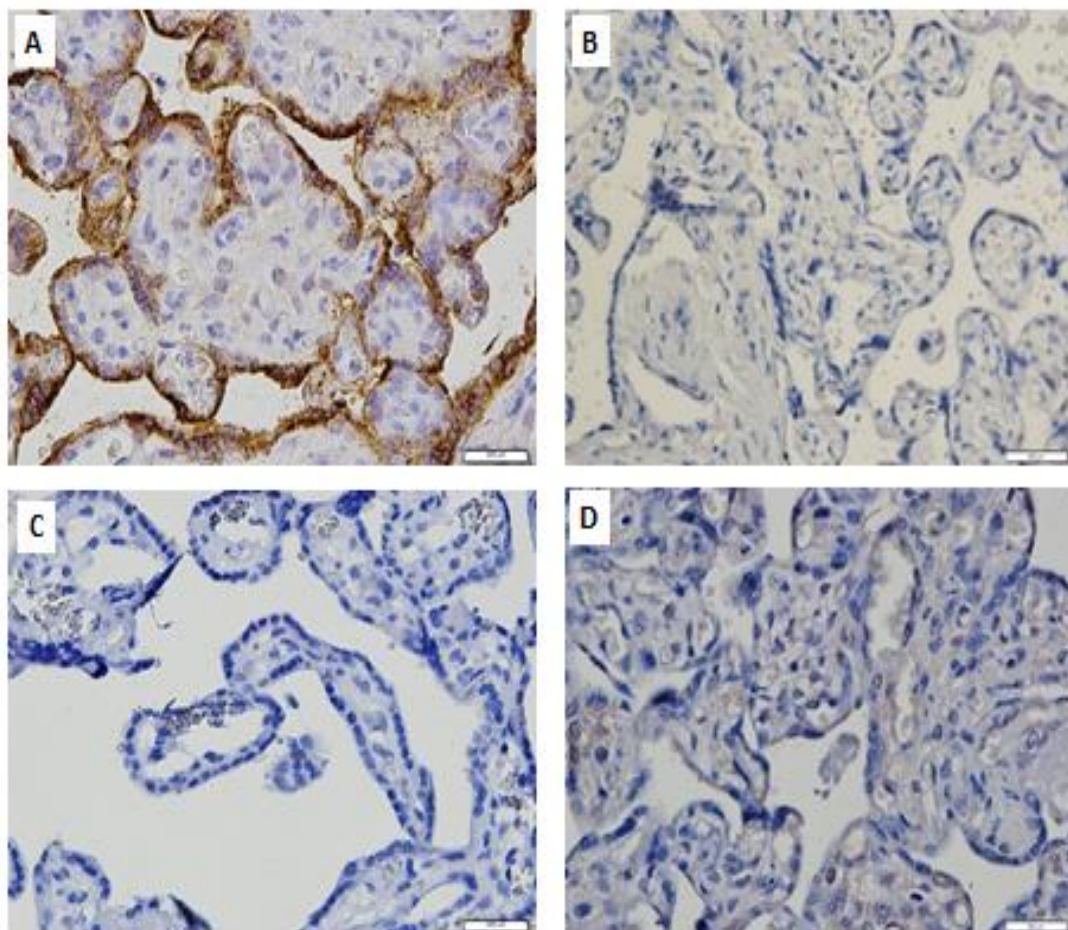
Detection of HPV16 L1 Protein

The productive infection by human papillomavirus is characterized by the expression of the late capsid protein (L1) which by itself can assemble into virus like particles (VLPs) (17, 28). Our PCR results (Figure 3.2) showed that both HIV positive and HIV negative placental tissues were positive for HPV16 by PCR of the L1 region. We therefore sought to determine whether L1 protein expression could be detected in placental trophoblasts. For this purpose we used a Mouse monoclonal anti-HPV16 L1 (H16.V5) antibody (49).

The trophoblast marker slide (Figure 3.7A) served as a reference for the location of the syncytiotrophoblast cells. To control for background staining due to non-specific binding, we used normal serum derived IgG4 isotype antibody. As expected, we did not observe any background staining (Figure 3.7B). Using an HIV-/HPV+ placental tissue, we also stained for L1 in the absence of the primary antibody and again, observed no detectable background signal (Figure 3.7B). There was no detectable L1 signal in the HIV-/HPV- placental tissue, (Figure 3.7D).

The HPV16 L1 protein was detected in HIV+/HPV16+ and HIV-/HPV16+ fetal placental trophoblasts (Figures 3.7E and F). Both cytotrophoblasts and syncytiotrophoblasts stained positive for the L1 protein, with most of the staining occurring along columns of syncytiotrophoblasts which are the terminally differentiated trophoblast cells. The L1 protein expression was also observed in the decidual cells of the maternal side of the placenta (Results not shown).

It has been postulated that the HIV-1 *tat* protein can transactivate the Long Control Region (LCR) of the HPV genome and upregulate the expression of E6 and E7 genes. Therefore, we chose to assess whether HIV could have an indirect effect on HPV16 L1 expression. We quantified the relative expression of L1 protein presence in placental tissue samples (Figure 3.7G). To achieve this, we used Image-Pro Premier Offline 9.0 which is software that we trained to discriminate between background signal and the actual HPV16 L1 signal. Subtraction of background signal gave the relative L1 signal per tissue area. The HPV16 L1 protein levels in all groups were determined in duplicate and the average L1 values, normalized to the tissue areas, were compared statistically. The median HPV16 L1 signal varied significantly (Kruskal Wallis: $p < 0.05$; $p = 0.0001$) across all the groups. The expression of the L1 protein was significantly different ($p < 0.05$; $p = 0.0231$) between the HIV-/HPV16+ and HIV+/HPV16+ groups. We concluded that expression of the L1 protein was clearly influenced by HIV status.



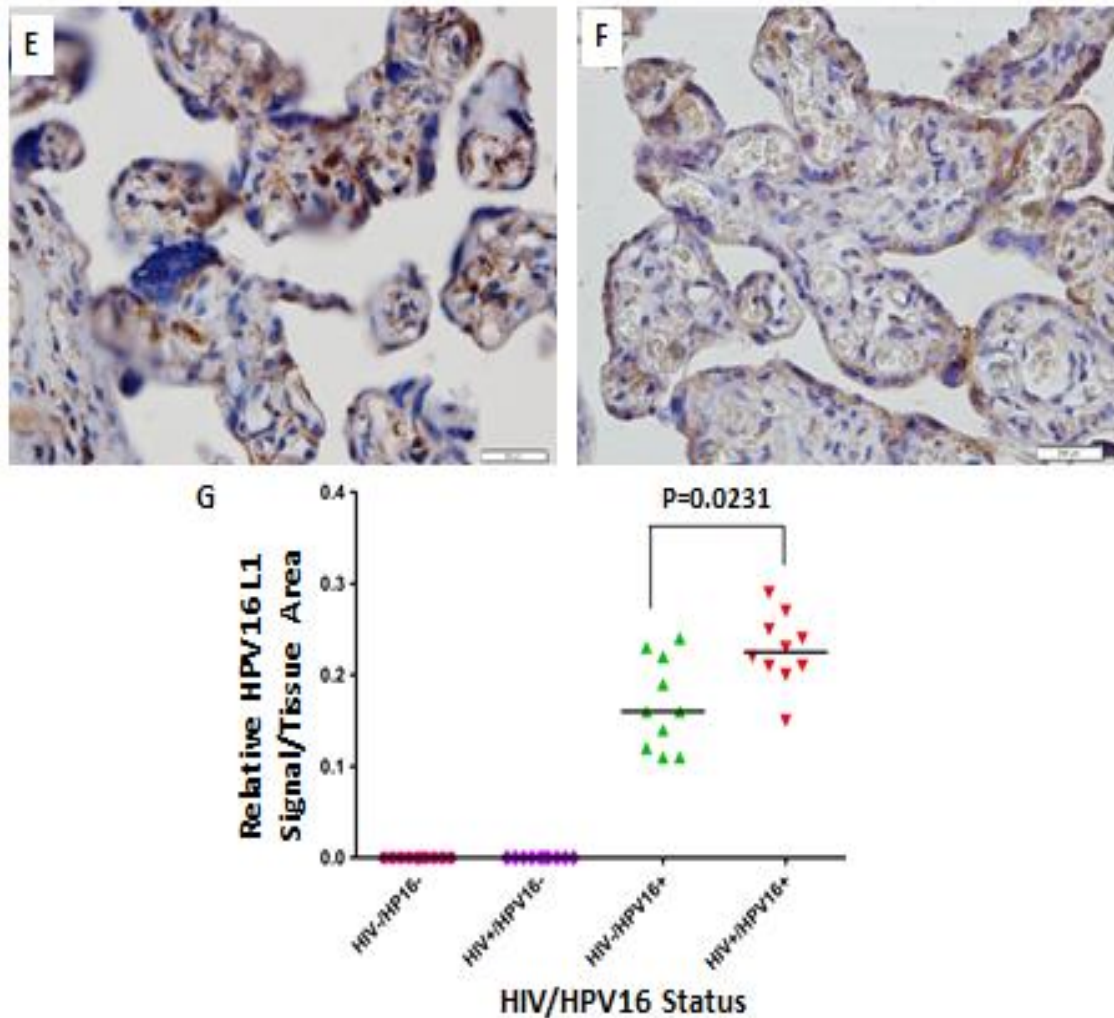


Figure 3.7 HPV16 L1 immunohistochemistry staining. (A) HSD3B1 trophoblast marker (B) Placental trophoblasts stained with a Mouse monoclonal anti-IgG isotype control. (C) Placental trophoblasts stained without a primary antibody. (D) Placental trophoblasts of an HIV-/HPV- tissue stained with anti-V5L1 antibody. (E) Placental trophoblasts of an HIV+/HPV16+ tissue stained with anti-V5L1 antibody (F) Placental trophoblasts of an HIV-/HPV16+ tissue stained with anti-V5L1 antibody (G) Quantification of HPV16 L1 protein in HIV-/HPV16+ and HIV+/HPV16+ sectioned placental tissues. Image Pro-Premier offline 9.0 was used to determine the HPV16 L1 relative levels in HIV-/HPV16+ and HIV+/HPV16+ sectioned placental tissues. The HPV16 L1 protein levels in all groups were determined in duplicate (Results not shown). The average HPV16 L1 values, normalized to the tissue areas, were compared using Kruskal-Wallis test. The bars represent the medians of the HPV16 L1 signal. The median HPV16 L1 signal varied significantly ($p < 0.05$; $p = 0.0001$) across all the groups. The expression of the L1 protein was significantly different ($p < 0.05$; $p = 0.0231$) between the HIV-/HPV16+ and HIV+/HPV16+ groups. We observed an HIV dependent effect on the expression of the L1 protein. As expected, all the HIV-/HPV16- and HIV+/HPV16- samples were negative for HPV16 L1 staining.

Discussion

Recent studies have demonstrated that HPV can infect epithelial trophoblast cells of the placenta (76) and HPV DNA has also been detected by PCR in placentas obtained trans-abdominally from women undergoing amniocentesis (71). These and other studies led us to explore the prevalence of HPV and the effect of HIV on HPV genotype distribution within the placental compartment.

To the best of our knowledge, we are the first group to study HPV infection of the placenta in the context of HIV infection. Using GP5+/6+ PCR as well as CPI/CPII primers, (Figure 3.2) we were able to detect HPV DNA in both HIV negative and HIV positive placental tissues.

Married HIV negative women were found to be placenta positive for HPV [73/81(90.1%)] as were married HIV positive women [75/95(78.9%)] as shown in Table 1. The high incidence of HPV infections among HIV+ and HIV- married women in this population could be explained in part by multiple sexual partners that they may have had by the time they were married as most of them are between 15 and 25 years. In low income countries such as Zambia, young women engage in sex earlier than do women in more affluent countries. Furthermore, there is a greater rate of women engaging in sex for monetary benefit, at a young age, which puts them at greater risk of contracting STDs, such as HPV and HIV. Placental samples were HPV6 positive at high rates, HIV+ (53%) and HIV- (69%), yet there was no significant difference ($p>0.05$) between the two groups (Figure3.4). We did not observe any significant differences between married HIV+ and HIV- women in most socio demographic status (Years of education and Household size) as a function of HPV genotype. Nevertheless, we observed a significant difference

(Fishers' Exact test: $p < 0.05$; $p < 0.0001$; 2-tailed) in HIV positive women aged 15-25 and 26-36 years [OR 0.0057, 95% CI, 0.000323-0.0992] when age was used as function of testing positive for HPV. HIV+ women aged 26-36 years were more at risk of testing positive for HPV than those aged 15-25 years. One plausible explanation for this observation is that women in this age group are most likely to have had multiple sexual partners, which put them at risk of being infected with both HIV and HPV.

The observed high HPV prevalence rate (Figure 3.3) in our study was not surprising because Zambia is endemic for both HIV and HPV (41). HPV prevalence rates as high as 97.2% have been previously reported among HIV positive Zambian women (51). To our surprise, we found that the prevalence of HPV in the HIV positive placental tissues [84/104(80.8%)] was slightly lower than that of the HIV negative ones [86/96(89.6%)], differing from previous studies (13, 23, 63). However, this difference in HPV prevalence between the HIV positive and HIV negative placental tissues was not significant (Fisher's exact test; $p > 0.05$; $p = 0.112$). Additionally, the prevalence rates for the two groups of placental tissues were calculated from different total numbers; that is, 86 out of 96 for the HIV negative group and 84 out of 104 for the HIV positive group.

In assessing the effect of HIV on HPV genotype distribution in the placental compartment, we found only three different types of HPV, namely the low risk HPV6, the high risk HPV16 and the rarely isolated HPV90 (Figure 3.4). We discovered that the incidence of the high-risk HPV16 in HIV positive placental tissues was greater than that in HIV negative tissues. There was a significant difference ($p < 0.05$; $p = 0.0241$) in the incidence of HPV16 between the two groups of placental tissues (Figure 3.4). We found that HIV positive placental tissues were two times more likely to harbor the high-risk

HPV16 than the HIV negative placental tissues. This result was in agreement with that obtained in a study conducted by Ng'andwe and colleagues in which they found a nine-fold increase in the incidence of the high risk HPV18 in HIV positive versus HIV negative vaginal lavage samples of Zambian women. It appears that the replication efficiency of the high risk HPVs is increased in patients whose immune system is compromised (41).

Interestingly, our placental HPV genotyping distribution differed from the vaginal lavage results that Ng'andwe et al had previously obtained from the same patients. This observation suggests that different HPVs could prefer different compartments (Table 3). In the previous vaginal lavage study by Ng'andwe and colleagues in our laboratory on the same patient samples (40) HPV16 and HPV18 were recovered in high abundance. This is in contrast to our present placental study in which HPV6 and 16 were the main genotypes isolated. Whereas in our study of the vaginal HPV distribution, we recovered 20 different HPV genotypes, the placental compartment was limited to only 3, suggesting very selective growth conditions in that tissue. Arguably, HPV6 appears to be the most successful at exploiting the placental compartment. It is important to acknowledge that differences in the rate of PCR detection of HPVs in different compartments could influence these results. The analysis of HPV in the vaginal and placental compartments was performed under exactly the same conditions using the same protocols. Second rounds of PCR for samples that tested negative for HPV was used to ensure that we did not leave out HPV positive samples.

Although HPV90 has been previously reported in an underserved population in United States, there is dearth of information to determine its prevalence, distribution and disease

association (44). The presence of HPV90 in cervical lesions as previously reported and now in placental tissues of our study samples may imply that this genotype has the potential to replicate in various epithelial compartments. To the best of our knowledge, we are the first group to report the presence of HPV90 in Zambian specimens and this finding suggests that unique HPV isolates may be present in this population.

We used p16 as a biomarker for HPV16 infection (38, 46, 65). To study the effect of HIV on p16 expression in HPV16+ and HPV16- tissues, we performed immunohistochemistry on tissue sections and stained them with a monoclonal anti-p16 antibody. Immunohistochemistry results from studies previously conducted on normal cervical tissues showed absence of p16 expression (65). In contrast, our p16 IHC trophoblastic results showed moderate expression of p16 both in the HIV-/HPV16- tissue (Figure 3.6C). One plausible explanation for this has been advanced by Tringler et al., 2004, who have previously demonstrated that p16 is expressed in normal villous cytotrophoblasts (CTB) between weeks 8 and 10; 15 and 18 as well as 37 and 39 of the gestational period. In their study, Tringler et al., 2004, observed a high p16 protein expression in the nuclei of normal CTB and extravillous (ETV) cells at 17 weeks of the gestational period. The high p16 expression kinetics was in tandem with the reshaping of the villous and was attributed to death of luminal epithelial cells and the decidua. The net effect of this has been observed, in a mouse model, to be decidualization of the endometrium and invasion of trophoblastic cells (65, 75). It is thought that p16 expression occurs in normal villous CTB in order to suppress villous proliferation and consequently promote the invasion process (65). Overall, the relative p16 expression median across all the 4 groups varied significantly (Kruskal-Wallis: $p < 0.05$; $p = 0.0367$) as shown in Figure 3.6F. We observed

a small difference in p16 expression between the HIV-/HPV16- and HIV+/HPV16- tissues, however, it was not significant (Mann-Whitney: $p > 0.05$; $p = 0.4836$; Figure 3.6F). This result suggests that HIV may have a mild effect on the expression of p16 in HPV-tissues. On the other hand, we observed a significant difference (Mann-Whitney: $p < 0.05$; $p = 0.0142$) in relative p16 expression between the HIV+/HPV16+ and HIV-/HPV16+ tissues (Figure 3.6F). This HIV-dependent effect on p16 expression could be attributed to a number of different indirect effects: that HIV can attenuate the cellular mediated immune responses (42) and also that the HIV-1 tat protein transactivates the HPV Long Control Region (LCR) causing an upregulation in the expression of the HPV18 E7 oncogene (64). The elevated E7 oncoprotein in turn binds to the E2F-pRb complex, causing the release of E2F and binds to pRb, which leads to induction of overexpression of p16 (38, 46, 65). Thus, the HIV-1 *tat* protein may influence HPV infection and increase p16 protein expression in trophoblastic cells. To determine whether this is possible *in vivo*, there is need to perform dual staining for HIV-1 tat and the HPV16 LCR.

In vitro studies have shown that the human papillomavirus can replicate productively in placental trophoblasts. In our study, we performed immunohistochemistry staining for the HPV16 L1 protein (Figure 3.7) using a monoclonal anti-HPV16 V5 L1 antibody (67) on patient-derived placental samples. The L1 protein is the major viral capsid protein that is expressed during the late phase of the HPV life cycle and can by itself spontaneously assemble into virus like particles (17, 28). Neither the HIV-/HPV- negative control (Figure 3.7D) nor did the anti-IgG4 isotype control (Figure 3.7B) give a positive signal for HPV16 L1. Additionally, the HIV+/HPV16+ tissue section stained without anti-L1

(Figure 3.7C) gave no detectable signal for HPV16 L1. We observed positive staining for L1 protein expression in HIV+/HPV16+ (Figure 3.7E) and HIV-/HPV16+ (Figure 3.7F) placental trophoblasts. The staining was observed both in cytotrophoblast and syncytiotrophoblast cells. It appears that HPV16 establishes its productive infection in these cells. To further elucidate this assertion, we wanted to perform electron Microscopy on the placental samples so that we could show the presence of HPV16 virion particles in these cells. This was however not possible because the membranes were disrupted during paraffin embedding of the placental tissues. Therefore as an alternative to this method, we intend to show evidence of the HPV16E1^{E4} splicing product in trophoblastic cells in our follow up study. Having identified the cells that were positive for HPV16 L1 staining, we quantified the relative HPV16 L1 protein using Pro-Premier Offline 9.0. We observed that the relative HPV16 L1 median signal varied significantly (Kruskal-Wallis: $p < 0.05$; $p = 0.0001$) across all the 4 groups (Figure 3.7G). The elevated expression of HPV16+ L1 in HIV+/HPV16+ trophoblastic cells in comparison to the HIV-/HPV16+ suggests that HIV has an effect on HPV16 L1 protein expression. To further explore this, we compared the HPV16 L1 relative signal between the HIV+/HPV16+ and HIV-/HPV16+ tissue samples (Figure 3.7G) and observed that there was a significant difference (Mann-Whitney: $p < 0.05$; $p = 0.0231$). The HIV dependent effect on HPV16 L1 expression in placental trophoblasts could be attributed to the ability of HIV-1 *tat* to transactivate the HPV LCR as alluded to earlier in the case of p16 expression (13, 64). Thus, our HPV16 L1 immunohistochemistry result corroborates the in vitro report that HPV can replicate productively in 3A trophoblasts in tissue culture (79).

Infection of the cervix with the HR-HPVs such as HPV16 and 18 are associated with cervical dysplasia (16, 83). Interestingly, we did not find obvious pathology in placental trophoblasts related to the presence of HR-HPV16. One plausible explanation for the absence of pathology in trophoblastic cells is that extravillous trophoblast cells express neither Major Histocompatibility Complex (MHC) class I nor class II molecules (22, 45). The lack of MHC classes I and II expression implies that the HPV virus can freely replicate without inducing an immune response.

Finally, in this study, we have shown the presence of HPV in placental trophoblasts using both polymerase chain reaction and HPV16 L1 immunohistochemistry methods. We have also shown, for the first time, the effect of HIV on HPV infection in placental trophoblasts.

In our follow up studies, we are determining the presence of HPV DNA in placental trophoblasts by *in situ* hybridization. Further corroboration of HPV infection of placental trophoblastic cells is being done by reverse transcription PCR for the E1^{E4} spliced product, which is the most highly expressed protein in productive infection by the human papillomaviruses. Additionally, we intend to perform HIV-1 p24 IHC using a polyclonal rabbit anti-p24 with a view to determining whether HIV is present in the placental cells. We further want to perform p24 staining using a monoclonal antibody in order to corroborate our results. This will be followed by dual-staining for HIV-1 p24 vs the cell markers, HIV-1 p24 vs HPV16 L1 and HIV-1 p24 vs HPV E1^{E4}. This will help us determine which cells are infected by HIV and HPV.

Finally, our main focus for this study was to determine the effect of HIV on high risk HPV16 infection of placental trophoblasts. We therefore now intend to perform

immunohistochemistry analysis for the low risk HPV6 and compare the results with those of HPV16 IHC. Overall, our results support the conclusion that a subset of HPVs infects the placenta and their prevalence is influenced by HIV status.

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