Role of Conserved Cysteine's and Methionine's

in

Human Papillomavirus-16 Major Capsid Protein L1

By

Sana Gul

2009-NUSTdir PhD-V&I-07

Atta-ur-Rahman School of Applied Biosciences
National University of Sciences & Technology
Islamabad, Pakistan
2016

Role of Conserved Cysteine's and Methionine's
in
Human Papillomavirus-16 Major Capsid Protein L1

By

Sana Gul

2009-NUSTdir PhD-V&I-07

A thesis submitted in partial fulfillment of the requirement for the degree of
Doctor of Philosophy
In

Healthcare Biotechnology

Atta-ur-Rahman School of Applied Biosciences
National University of Sciences & Technology
Islamabad, Pakistan
2016

Dedicated

To

Women of Pakistan

ACKNOWLEDGEMENTS

I start this acknowledgement with the name of Allah the most merciful, omnipotent and omnipresent, who has made me energetic enough to accomplish this task with enough concentration and devotion, and finally blessed me with success. I offer my humblest thanks from the core of my heart to the Holy Prophet Hazrat Mohammad (SAW) who guided the mankind to the path of righteousness.

I deem my utmost pleasure to avail the opportunity to express the heartiest gratitude and deep sense of obligation to my Supervisor Dr. Aneela Javed for her dexterous guidance, untiring help, kind behavior, moral support and enlightened supervision during the study period.

I promulgate scrupulous respect to principal of Atta-ur-rehman school of applied biosciences (ASAB); Dr. Peter John for his untiring effort in providing congenial environment for education and research.

Being a NUST Scholar, I am thankful to NUST for giving me scholarship for completion of this study.

I would also like to express my regards and sincere thanks to my GEC members Dr. Aneesa Sultan; Assistant Professor, QAU, Islamabad, Dr. Muhammad Tahir; Assistant Professor ASAB, NUST and Dr. Sobia Manzoor; Assistant Professor ASAB, NUST for their supporting behavior , guidance and invaluable suggestions.

I would like to thank my Mother and my Father from the core of my heart, for their unlimited efforts, they made for my wellbeing, for their patience and unfathomable love.

I would also thank my siblings too, love you all. I pay my heartfelt thanks to Dr. Craig Meyers, Dr. Samina Alam, Dr. Muhammad Israr, Janice Milici, Dr. Eric Rayndock, Dr. Jenn Biryukof and Dr Linda Cruz for their kindness and guiding behavior in research work.

I would also thanks to my precious friend Mamoona Noreen for their moral support and help during entire duration of studies.

I would also like to recognize the silent efforts of all of my colleagues around, whose un-noticed presence and quiet participation have always been at my back in one way or another.

SANA GUL

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
AD	Adenocarcinoma
B	Benzonase-treated
Bam	*BamHI*-treated
BD	Becton Dickinson
BPV	Bovine papillomavirus
CaCl2	Calcium chloride
CIN	Cervical Intraepithelial Neoplasia
CVP	Crude viral prep
Da	Dalton
DACM	*N*-[7-dimethylamino-4-methyl-3-coumaring]-maleimide
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithio-bis-[2-nitrobenzoic acid (Ellman's Reagent)
E	Early gene/protein
EDTA	Ethylene di-amine tetra acetic acid
EGCG	Epigallocatechin gallate
Endo	Endonuclease
EtOH	Ethanol
H&E	Hematoxylin and eosin
HFK	Human foreskin keratinocyte
HSIL	High grade squamous intraepithelial lesion

HPV	Human papillomavirus
IARC	International Agency for Research on Cancer
ICO	Information Centre on HPV and cancer
kD	Kilodalton
L	Late gene/protein
LC	Liquid chromatography
LSIL	Low grade squamous intraepithelial lesion
M	Mock
MAb	Monoclonal antibody
MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Nano
NB	Non-benzonase-treated
NEB	New England Biolabs
NEM	N-ethylmaleimide
NLS	Nuclear localization signal
ORF	Open reading frame
OTNV	Organotypic culture-derived native virion
pBS	Bluescript plasmid
PCR	Polymerase chain reaction

PsV	Pseudovirion
p-XSC	1,4-phenylenebis(methylene) selenocyanate
qPCR	Quantitative polymerase chain reaction
QV	Quasivirion
rC	Recombinant core
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCC	Squamous Cell carcinoma
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S-M	Selenomethionine
SV40	Simian virus 40
TE	Tris EDTA buffer
TEM	Transmission electron microscopy
URR	Upstream regulatory region
Vge	Viral genome equivalent
VLP	Virus-like particle
WT	Wild-type
10-d	10-day-old organotypic tissue
20-d	20-day-old organotypic tissue

±	Plus minus
µ	Micro
2-ME	2-mercaptoethanol

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Human Papillomavirus (HPV) is tumor inducing virus which infects mucosa and cutaneous keratinocytes. HPV is the causative agent for cervical cancer. The cervical cancer induced death rate is high among women in developing countries. There are various cancer causing subtypes of HPVs. The first aim of the current study was to find the most prevalent HPV subtype among the local cervical cancer patients. For that purpose, samples were collected from the patients of different ethnic and social back grounds (mostly from the twin cities Islamabad & Rawalpindi) visiting a Government tertiary care hospital between 2010-2012. A total of 67 formalin fixed paraffin-embedded sections of cervical lesions were obtained. PCR-based detection method showed the presence of HPV DNA in 59 samples. HPV16 was present in 30 samples, while 22 samples were positive for HPV18. A small number of HPV positive samples (7) were found to be HPV16 and HPV18 negative, representing a minor percentage (10%). However HPV16 and HPV18 accounted for 44.8% and 32.8% of samples respectively.

In the absence of therapy for an established HPV infection, there is a huge reliance on prophylaxis. The available prophylactic vaccines are mainly based on the self-assembly of the major capsid protein L1 into a virion-like icosahedral particle. After disruption of the intermolecular disulphide bonds, each icosahedral particle, dissociates into 72 pentameric capsomeres. Earlier, it has been proposed that C161, C229, and C379 may be necessary for the integrity of L1-capsids probably through intramolecular bonding. Therefore, the next objective of the current study was to explore the potential role of C161, C229, and C379 residues in HPV16 genome assembly, encapsidation and infectivity using organotypic tissue culture.

In order to do so cysteine residues; C161, C229, and C379 were mutated to serine through site directed mutagenesis. Linearized DNA from wild type and mutant HPV16 genome was electroporated in Human forskin keratinocytes (HFK's) and respective organotypic raft cultures were established. The comparison of crude viral preps from mutant and wild type HPV16 showed that endonuclease-resistant genomes in mutant viral preps was lower than that of wild type. It may be concluded that C161S, C229S and C379S are critical for proper formation of endonuclease resistant capsids. Furthermore, these mutants were found to cause 50% or more reduction in viral infectivity. To summarize, this study contribute to the current understanding about the conserved cysteine residues; C161, C229, and C379 and upstream methionine residues present in major capsid protein L1 in HPV16, maturation and more importantly in viral infectivity, also showing importance of using organotypic raft culture for studies.

Next objective was to determine the role of two upstream methionine residues in the biology of HPV16L1, within the natural environment of Human epithelial tissue. In order to do so, HFK's were electroporated with HPV16L1 methionine mutant 1(Met 1 with translational initiation at first methionine) and HPV16L1 methionine mutant 2 (Met 2 with translational initiation at second methionine). The infectivity assay showed higher infectivity in Met 1 as compared to Met 2.

Chapter 1

Introduction

Cervical cancer is the most common type of cancer in the women. Approximately more than 80% of these cases and deaths occur in developing countries because of lack of awareness and poor medical facilities (Walbommers *et al.,* 1999). Human papillomavirus (HPV) is the etiological agent for the development of cervical cancer. The very strong association between cervical cancer and HPV has been confirmed since 1976 (Burd, 2003; Bosch *et al.*, 2002). Two main types of cervical cancers are squamous cell carcinoma (SCC) and adenocarcinoma (AC). According to the International Agency for Research on Cancer (IARC-WHO) cervical cancer is the fourth most common cancer among women. During year 2012 there were estimated 528,000 new cases and 266,000 deaths worldwide because of cervical cancer (Globocan, 2012). Regular cervical screening and HPV vaccines has resulted in a dramatic decline in cervical cancer burden in developed countries. Unfortunately, in developing countries cervical cancer remains one of the leading cause of death among women (de Sanjose *et al*., 2010). Cervical cancer is more prevalent in Asia where it records around half of the world's new cases of cervical cancer each year (Lyn, 2014). Pakistan is an underdeveloped country and screening of HPV in Pakistan is not normally implemented. No concrete data is available in relation to the population prevalence or risk factors for HPV infection in Pakistan. Only institutional and regional cancer registry provides some information about cervical cancer, which is often incomplete (Aziz *et al* 2003; Badar *et al* 2007; Bhurgri, 2004). Attaining the data regarding HPV associated cervical cancer will help to develop policies regarding the need for the HPV prophylactic vaccination and screening programs in the country.

HPVs are epithelial viruses and more than 200 HPV types have been discovered up till now. HPV has the ability to infect the mucosal and cutaneous keratinocytes. HPV types that infect mucosa can be further divided into high-risk and low risk HPV types. High-risk types can develop malignant neoplasm for example cervical cancer and Low-risk HPV types can cause benign lesions (de Villiers *et al.,* 2004; Longworth *et al.,* 2004). About 70% of cervical cancer is because of High-risk HPV strains such as HPV 16 and 18 while 20% of cervical cancer shows an association with other high risk HPV genotypes. High-risk HPV sub-types such as HPV16, HPV18, HPV45 and HPV31 are often found to be associated with the malignant progression (de Villiers *et al* 2004).

HPVs are non-enveloped with icosahedral capsids. Genome size of viral HPV is approximately 8 kb in size. Within the capsids, viral genomes forms linkage with cellular histone's and forms chromatin like structure (Doorbar 2005; Larsen *et al* 1987). HPV viral genomes consist of eight genes and all of them are expressed from polycistronic mRNAs which are transcribed from single DNA stand (Favre *et al* 1975; Zheng and Baker 2006). All eight genes are further divided in to early (E) and late (L) genes. There is also an upstream regulatory region (URR) which is also known as non-coding region (Buck *et al* 2008; Zheng and Baker 2006).

The early (E) genes consist of the E1, E2, E4 E5, E6, and E7 genes. E1, E2, E4 E5, E6, and E7 proteins are encoded by their respective genes (Buck *et al* 2008; Zheng and Baker 2006; Doorbar *et al* 1986). E1^E4 spliced protein plays role in distressing the viral genomes replication and inhibit switching of G2-M state. These proteins collectively collapse the cellular keratin networks, thus helping virions to escape the cell (Brown *et al* 2006; Bryan and brown 2000; Gambhira *et al* 2007; Knight *et al* 2006; Wang *et al* 2004; Wilson and laimins 2005). Other early proteins such as E5, E6 and E7, encoded by the high-risk HPVs are reported to oncogenic with

the ability to change the cell morphology and induce cellular proliferation of the infected cells (Androphy *et al* 1987; Bedell *et al* 1991; Phelps *et al* 1988). L1 is a major capsid protein and L2 is minor capsid protein. L1 and L2 proteins are encoded by L1 and L2 genes respectively (Buck *et al* 2008; Chen *et al* 2000; Doorbar and Gallimore1987; Favre 1975).

HPV life cycle depends on the differentiation process of host epithelial tissue. Mature virions assemble in terminally differentiated suprabasal cells (Barksdale and Bakers 1993; Hummel *et al* 1992; Meyers *et al* 1992). Micro-abrasions of epithelial tissue is considered the preliminary entry site for HPV virions through which HPV enters in to the basal layer of cells (Belnap *et al* 1996). HPV entry receptors are not confirmed, two receptors alpha integrin and heparin sulfate have been suggested as possible entry receptors for HPV entry (Bousarghin *et al* 2003; Day *et al* 2003; Fligge *et al* 2001; Hindmarsh and Laminis, 2007; Smith *et al* 2007). There is limited data in relation to the exact mechanism responsible for the delivery of viral genome in the nucleus of host cell (Smith *et al* 2007).

Two major viral promoters initiate the transcription of high-risk HPV (Conger *et al* 1999; Meyers *et al* 1992; Mohr *et al* 1990; Smith *et al* 2007). The promoter for early genes is upstream of the E6 open reading frame (ORF) and produces transcript, this promotor is called as p97 (Mohr *et al* 1990).

Late promoter initiation takes place in a differentiation-dependent manner. In HPV16 p670 is identified as late promoter (Barksdale and Baker 1993). The function of the late promoter is to generate a transcript that translates in to L1 and L2 proteins (Fig. 2.4). E1^E4, and E5 are early proteins and they are translated in a differentiation-dependent manner. E1^E4, and E5 proteins can influence the replication of viral genomes. E1^E4 inhibit the transition of G2-to-M and they

also affect E5 by stimulating cell cycle development (Bird *et al* 2008; Buck *et al* 2004; Chromy *et al* 2006; Chromy *et al* 2003).

It is clear from studies that when capsid proteins are expressed, they assemble in to icosahedral structure with the help of chaperone proteins in proper redox environment (Bird *et al* 2008; Bordeaux *et al* 2006; Buck *et al* 2008; chromy *et al* 2006; chromy *et al* 2003; Doorbar and Gallimore 1987; Fligge *et al* 2001; Heino and Lambert 2000; Zhao *et al* 2000).

Capsid of papillomavirus are made up of various components; for example viral DNA, histones, L1 and L2. Cellular components in addition to viral components could contribute towards the development or yield of capsids (i.e. molecular chaperones, karyopherins) either directly or indirectly (Bryan and Brown 2001). For proper assembly of native virion some additional unknown viral or cellular factors are needed which helps in the formation of correct disulfide bonds and also help in cellular positioning and early contact of capsid proteins and expression control of capsid protein. Mature virions escape from desquamated cornified cells with the help of E1^E4 proteins. These proteins interact with keratin networks of host cells, causing their collapse and allowing the virions to escape (Chen *et al* 2000; Hagensee *et al* 1994; Modis *et al* 2002). It remains unclear what molecular interactions regulate the monomeric capsid proteins to assemble into complete structures during host cell keratinocyte development. In order to understand different phases of life cycle of HPV such as HPV infectivity, its immunogenicity, route of transmission and structure of virus, recent research use various *in vitro* systems. These *in vitro* systems are based on recombinant papillomavirus particles such as virus-like particles (VLPs), quasivirions (QV) a papillomavirus containing genome, (PsV) pseudovirions a papillomavirus based on gene transfer vectors. All these systems bypass the need of natural host environment for the production of native HPV. In order to generate recombinant papillomavirus

particles in stratifying and differentiating human epithelial tissue, the human forskin-based organotypic culture (xenograft), or native tissue cultures are the closest to *in vivo* HPV infection.

Chapter 2

REVIEW OF LITERATURE

CERVICAL CANCER

Globally cervical cancer is accepted as second common type of gynecological cancer among women, more than 80% of incidences and related deaths occur in developing countries (Walbommers *et al.,* 1999). In almost all cases (99.7%) of cervical cancer, Human papillomaviruses (HPVs) are the etiological agents for the development of cervical cancer. A very strong association between cancer and high risk HPV types 16, 18, 31, 45, etc. (de Villiers *et al.,* 2004; Longworth *et al.,* 2004) has been confirmed since 1976 (Burd, 2003; Bosch *et al.,* 2002). Two main types of cervical cancers are squamous cell carcinoma (SCC) and adenocarcinoma. The most common cervical cancer is SCC and more or less 90% to 95% of all the cervical cancers are SCC. The abnormal growth of cells in the cervix can be detected through cervical screenings such as pap smear and cervical colposcopy. These are called squamous intraepithelial lesions (SIL) and can be graded depending on the disease severity. There are two different types of SIL

- High grade squamous intraepithelial lesion (HSIL)
- Low grade squamous intraepithelial lesion (LSIL)

In most of the women it takes several years to develop cervical intraepithelial neoplasia 3 (CIN3) from a normal cervix. Not all women with CIN3 will go on to develop invasive carcinoma (McIndoe *et al*., 1984). According to the International Agency for Research on Cancer (IARC-WHO) Cervical cancer is the fourth most common cancer among women, during year 2012 there were estimated 528,000 new cases and 266,000 deaths worldwide because of cervical cancer (

Globocan, 2012). Burden of cervical cancer has reduced in developed countries by over 70 percent due to regular cervical screenings and HPV vaccination. Still cervical cancer is one of the major causes of death among women in developing countries (de Sanjose *et al*., 2010). Cervical cancer is more prevalent in Asia where it records around half of the world's new cases of cervical cancer each year, this situation is because of lack of awareness, poor medical facilities, lack of access to good medical services, and costly vaccination. Cervical cancer cases are underestimated by 40 to 50 percent because unfortunately all cancer cases are not registered (Lyn, 2014). Pakistan is an underdeveloped country and screening of HPV in Pakistan in general is not implemented. There is lack of documented data in relation to the HPV associated cervical cancer in local patients. Only institutional and regional cancer registries provides some information about cervical cancer, which may not be true representation of cervical cancer burden. Therefore, there is deficient epidemiological data regarding HPV. These registries only represent the cervical cancer prevalence rate but lacks HPV associated information. Furthermore, there are patients from remote areas with little or no medical facilities, cannot be diagnosed and treated for the disease (Aziz *et al* 2003; Badar *et al* 2007; Bhurgri, 2004). According to Information center on HPV and cancer (ICO), in Pakistan cervical cancer ranks as the 3rd most common cancer among women. Every year in Pakistan 5233 women are diagnosed with cervical cancer while 2876 die of the disease (ICO, 2015). Gathering HPV related data is important in order to lower the burden of cervical cancer and also help to check the potential relevance of HPV vaccination.

Human Papillomavirus

More than 200 HPV types have been discovered till now. HPVs are epithelial viruses and have ability to transmit a disease to mucosal and cutaneous keratinocytes. HPV types that infect mucosa can be further divided into high-risk and low risk HPV types. High-risk types can develop into malignant neoplasm such as cervical cancer and Low-risk HPV types can develop into benign neoplasms (de Villiers *et al.,* 2004; Longworth *et al.,* 2004). High risk HPV types such as HPV16, HPV18, HPV45 and HPV31 shows association with malignant development (de Villiers *et al* 2004). About 70% of cervical cancer is because of High-risk HPVs such as HPV 16 and 18 while 20% of cervical cancer shows an association with other high risk HPV genotypes. High Risk HPV16 is the most common HPV type found in women with normal cytology as well as with cervical lesions or cancer, followed by HPV-18 in central and South America. HPV type 53 and 52 is common in North America and HPV-52 & HPV-58 in Asia. According to International Association for Research in Cancer (IARC), the most common high risk genotypes which transmit a disease to the cervix are: HPV-16 (53%), HPV-18 (15%), HPV-45 (9%), HPV-31 (6%), and HPV-33 (3%) (Munoz, 2000). The precise data about the most prevalent high-risk genotypes is essential for the development of more efficient screening programs, management of the cancers related to HPV especially cervical cancer and development of vaccine against the specific prevalent genotypes.

Propagation and Analysis of HPV *In vitro* and *In vivo*

HPV life cycle dependence on stratifying and differentiating human epithelial tissue has made *in vitro* analysis and propagation of the HPV quite challenging. While different methods exist to study HPV life cycle in the environment of its natural host of human epithelial tissue. There are certain methods like recombinant, monolayer culture-derived papillomavirus particles that avoid

the production of HPV in natural host environment (Buck *et al.,* 2008; Buck *et al.,* 2005, Campos *et al* 2009; Culp *et al* 2004; Culp *et al* 2006; Day *et al* 2003; Kirnbauer *et al* 1992; Kirnbauer *et al* 1993; Pastrana *et al* 2005; Roden *et al* 1997; Smith *et al* 2007; Smith *et al* 2008). Advanced technologies allow the production of the recombinant papillomavirus particles outside epithelial tissue, but there are limitations with these technologies. In order to fully understand the detailed steps involved in the papillomavirus life cycle, recombinant papillomavirus particles need to be produced in the stratifying and differentiating epithelial tissue.

Skin composition

Skin is made up of two layers. From inside to outside the body, the skin is composed of dermal and epidermal layers. Skin's epidermal layer, sometimes called a stratified epithelium, contains multiple sub-layers. Skin is also facilitated by a hypodermal layer (also called subcutaneous tissue) (Querleux *et al* 2002). Although the hypodermal layer is not technically part of skin, it provides critical support to the dermis and stratified epithelium – attaching skin to underlying bone and musculature in addition to providing sufficient connections to the circulatory and nervous systems (Querleux *et al* 2002). The hypodermis is mostly made up of loose connective tissue and elastin, embedded with fibroblasts, macrophage, and a large percentage of adipocytes (Querleux *et al* 2002). The dermal layer lies directly above the hypodermal layer and below the stratified epithelium (Burgeson and Cristiano, 1997). The region closest to the hypodermal layer is thick and is called the reticular region whereas the region closest to the epidermal layer is less thick and is called the papillary region (Burgeson and Cristiano, 1997). The dermis as a whole consists of connective tissue embedded with sweat glands, hair follicles, apocrine glands, sebaceous glands, lymphatic vessels, and blood vessels (Burgeson and Cristiano, 1997). The dermal layer is closed to the basement membrane of the stratified epithelium and its embedded

blood vessels facilitate waste removal from the lowest sub-layer of the epithelium, the stratum basal (Fig. 2.1) (Burgeson and Cristiano, 1997, Madison 2003).

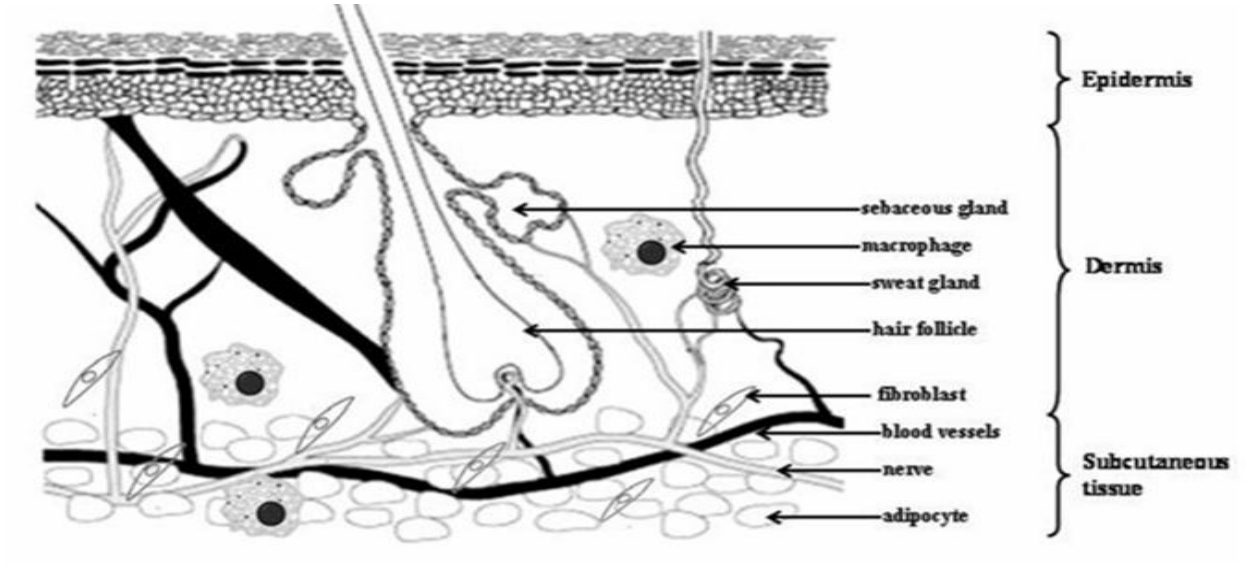

Figure 2.1 Cross-section of human skin and subcutaneous tissue (adapted from Madison 2003) The two layers of human skin are the dermis and epidermis. Skin's epidermal layer contains multiple sub-layers. The bottom layer of the epidermis is the stratum basale or basal layer. The basal layer makes contact with the dermal layer. The dermis consists of connective tissue embedded with hair follicles, sweat glands, sebaceous glands, blood vessels, and other components. Skin is also reinforced by a critical hypodermal layer, which is also called subcutaneous tissue

.

The skin's epithelium is composed of five sub-layers. From inside the body to outside, the sub-layers are the: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and the stratum corneum (Madison 2003). These layers or strata are formed as stem and transit amplifying cells within the stratum basale undergo mitosis, forcing daughter cells up through the various strata as they continue to divide (Bastein and McBride, 2000; Fuchs 1990; You *et al* 2004). The stratified epithelium is made predominantly of keratinocytes a cell type that is characterized by the expression of cytokeratin proteins (Fuchs 1990). Cytokeratins are members of a large family of intermediate filament proteins that form heteropolymers of basic and acidic cytokeratins. These polymers make up the cytoskeletal framework of keratinocytes (Jorcano *et al* 1984, Mischke 1998). As these cells migrate through the various strata, molecular signals are received that induce a program of terminal differentiation, whereby, the cells begin to change shape (flattening and stretching out) and fill with a network of cytokeratin filaments (Eckert *et al* 1997, Madison, 2003). Before terminal differentiation, keratinocytes lose the ability to go through the cell cycle and they undergo nuclear disintegration (Broekaert *et al* 1986). Terminally differentiated keratinocytes are called squames. Squames are desquamated or sloughed off the epithelium only to be replaced by a new generation of daughter cells that migrate through the strata, to become a squame (Eckert *et al* 1997). This entire cycle of squamous epithelial tissue takes approximately two weeks to complete (Mischke, 1998).

Epithelial stratification and differentiation. In normal stratified epithelial tissues, the stratum basale is the only layer that contains actively dividing cells, called basal cells (Fuchs, 1990). The exception would be in preneoplastic or neoplastic epithelial tissues where cell growth is uncontrolled (Glick *et al* 1993). Basal cells are round in shape compared to cells in the upper strata and predominantly express cytokeratins 5 and 14 (Fuchs 1990; Fuchs and Cleveland 1998; Mischke 1998). Unknown signals influence the migration of daughter cells from the actively

dividing basal cells into the upper layers. Above the stratum basale, cells within the stratum spinosum are embedded with desmosomes (Eckert *et al* 1997). Desmosomes facilitate cell-to-cell adhesion and resist shearing forces between cells (Eckert *et al* 1997). In addition to the appearance of desmosomes, differentiation-specific proteins including involucrin and transglutaminase are expressed (Eckert *et al* 1997; Eckert *et al* 1993; Kalinin *et al* 2003). Involucrin forms a scaffold which allows for the early deposition of cornified envelope components, and transglutaminase catalyzes the formation of peptide bonds between involucrin, loricrin, small proline-rich protein and other cytokeratin proteins during this process (Candi *et al* 2005; Eckert *et al* 1997; Eckert *et al* 1993; Rice and Green 1978). The cornified envelope replaces the cell membrane, converting it into a cytokeratin-rich amalgam of proteins and lipids (Candi *et al* 2005). At this point in the differentiation process, the transcription of stratum basalespecific cytokeratins is turned off and cytokeratins 1 and 10 are expressed (Fuchs 1990; Kalinin *et al* 2001).Within the stratum spinosum, cellular DNA replication ceases, and cell cycle machinery is downregulated through the upregulation of tumor suppressors and other inhibitors of the cell cycle in addition to the enhanced expression of microRNA-203 which inhibits p63 – a regulator of stem cell maintenance (Gosselet *et al* 2007; Hauser *et al* 1998; Lopez *et al* 2009; Martinez *et al* 1999; Yi *et al* 2008). Above the stratum spinosum, the stratum granulosum contains cytoplasmic granules that contain loricrin and profillagrin (Eckert *et al* 1997; Kalinin *et al* 2001). Loricrin is a component of the cornified envelope, whereas, profilaggrin is proteolyzed into filaggrin which bundles keratin filaments into larger tono filaments (Eckert *et al* 1997; Kalinin *et al* 2001). In addition to protein, differentiation-dependent enzymes are involved in the processing of lipid precursors within organelles called lamellar granules. Lipid moieties such as ceramides, free fatty acids, and cholesterol sulfate are also deposited at junctions between cells to preserve moisture within the skin (Choung *et al* 2002; Madison, 2003). To do this, lipid-filled

lamellar granules fuse with the plasma membrane at the interface between the stratum granulosum and stratum corneum allowing for the deposition of the lipids within the lamellar granules into a growing lipid envelope (Candi *et al* 2005; Choung *et al* 2002; Madison, 2003). In the stratum corneum, the cells eventually die, lose their organelles as they are filled with cytokeratins and lipids persist only until mechanical action facilitates their sloughing off into the environment (Fig. 2.2) (Candi *et al* 2005; Choung *et al* 2002; Eckert *et al* 1997; Madison, 2003)

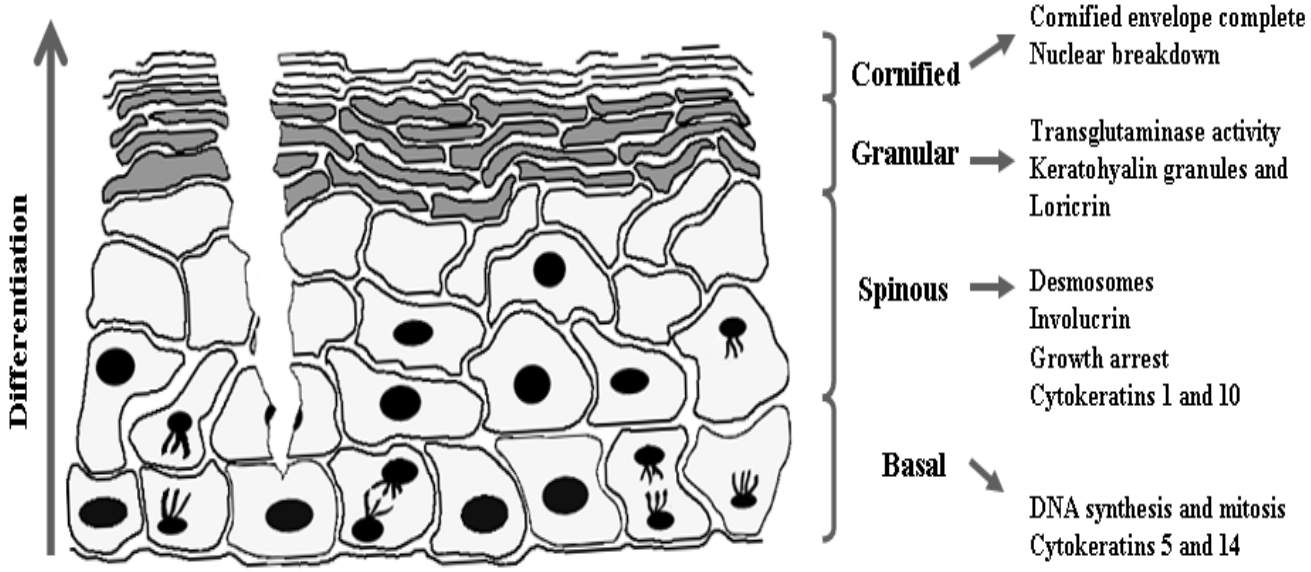

Figure 2.2. 2D model of human epithelial tissue stratification and differentiation. Adapted from Candi *et al* 2005.

Stratified epithelial tissue can be sub-divided into four general layers from the inside of the body to the outside of the body: stratum basale (basal), stratum spinosum (spinous), stratum granulosum (granular), and stratum corneum (cornified). The basal layer is the least differentiated layer whereby DNA synthesis and mitotic activity still occurs. In this layer, cytokeratins 5 and 14 are expressed. The spinous layer is characterized by the presence of desmosomes, involucrin, growth arrest of cells, and the expression of cytokeratins 1 and 10. The

expression of cytokeratins 5 and 14 are downregulated. The granular layer contains transglutaminase activity and also contains keratohyalin granules and loricrin. The cornfied layer consists of fully differentiated cells that have flattened and stretched out called squames. Squames have a complete cornified envelope and fully dissolved nucleus. A microabrasion that tears through the stratified epithelium, exposing the stratum basale is shown on the left (candi *et al*., 2005).

Different models of invitro epithelial differentiation

A variety of methods including cell suspension, confluency, high calcium, high serum, and organotypic raft culture have been developed to induce the differentiation of isolated keratinocytes (Bedell *et al* 1991; Lee *et al* 1998; Matsui *et al* 1992; Meyers *et al* 1997; Pillai *et al* 1991; Poumay and Mleclereq, 1998; Prunieras *et al* 1983; Wilson and Laimins, 2005). None of these techniques perfectly recapitulates the *in vivo* environment that is integrated into the circulatory system, nervous system, lymphatic system; however they remain simple tools to dissect the complexity of epithelial tissue differentiation. Suspension of cells as a means to induce differentiation appears to mimic the detachment of stratum basale-bound cells, which is a requisite for migration into the upper epithelial strata. Mimicking this process by growing cells in suspension can induce differentiation in individual keratinocytes. In the case of human foreskin keratinocytes (HFKs), if suspended in 1.6% methylcellulose, they lose colony formation and re-attachment abilities, followed by a loss of cyclin A, increase in p27, involucrin, cytokeratins 1 and 10 and an increase in cornified envelope production in many of the cells (Ruesch *et al* 1998; Wilson and Laimins, 2005). In the case of 9E cells, a subclone derived from an HPV31b-infected CIN-612 cell line obtained from a CIN I lesion from a 22 year old woman., In this, nearly all cells post-methylcellulose suspension stain positive for involucrin and

transglutaminase within 24 hours (Meyers *et al* 1992; Ozbun 2002; Ozbun and Meyers, 1997; Ozbun and Meyers, 1998 (a); Ozbun and Meyers, 1998 (b)).

Cytokeratin 10 expression is induced slightly and expression of filaggrin is poor. Because of these differentiation markers, it appears that 9E cells reach a level of differentiation that is similar to cells within the stratum spinosum. Although protein markers indicate that cells in suspension are undergoing a process of differentiation similar to what is observed in biopsy-derived tissue, cells in suspension never flatten and strech out as squames, and only a small proportion undergoes nuclear dissolution (Wilson and Laimins, 2005). Although it is not usually recommended cell culture practice, but growing keratinocytes to confluency induces the expression of granular markers (Lee *et al* 1998; Poumay and Mleclereq, 1998; Ura *et al* 2004). Before confluency, keratinocytes are clonogenic and remain as a monolayer, but they lose this property upon entering into a high cell density environment (Poumay and Mleclereq, 1998; Ura *et al* 2004). Here, layers of cells can be observed in a confluent plate of cells whereby cytokeratin 1 and 10 are induced, and it has been proposed that the level of confluency of a plate of keratinocytes can alter the level of differentiation reached upon switching to suspension culture (Poumay and Mleclereq, 1998). A variety of methods such as the addition of detergents, NaCl, and ionophores, increase the permeability of keratinocytes to exogenous calcium or raise the total level of calcium within the cell (Pillai *et al* 1991). Such treatments enhance desmosome and other protein marker expression such as cytokeratin 10, resulting in the inhibition of cellular DNA replication and development of cornified envelopes (Matsui *et al* 1992).

Organotypic raft culture: In comparison to the rest of the culturing techniques organotypic raft culture is the only technique that allows for the *in vitro* development of a fully differentiated multicellular epithelial tissue. It should be noted that a variety of techniques and materials allow the production of organotypic raft cultures in the laboratory, however, all include the growth of

isolated keratinocytes on a synthetic dermal equivalent infused with mouse fibroblasts at the air-liquid interface (Holmgren 2005; Mclaughlin *et al* 2004; Meyers *et al* 1992; Ozbun and Meyers, 1998; Patterson *et al* 2005; Pyeon *et al* 2005; Wang *et al* 2004). Exposure to air and the filtering action of the dermal equivalent have been proposed as key factors determining proper stratification and differentiation of the epithelial tissue (Prunieras *et al* 1983). Importantly, the morphology of organotypic tissues is very similar to the morphology of biopsy-derived tissue (Prunieras *et al* 1983). However, some protein markers suggest that organotypic culture-derived tissues represent tissue from a wound healing environment or from psoriasis, rather than normal, healthy tissue (Asselineau *et al* 1986). Even so, all strata of the epidermis are represented, and organotypic raft culture technique is the only other than xenograft systems that permit the development of infectious HPV in the natural host environment that is stratifying and differentiating epithelial tissue (Mclaughlin *et al* 2004; Mclaughlin *et al* 2005; Meyers *et al* 1992; Meyers *et al* 1997). Importantly, in the organotypic culture system, HPV genomes can be mutated, electroporated into non-infected keratinocytes, and utilized to determine the role of HPV genomic and proteomic elements in the natural life cycle of the virus.

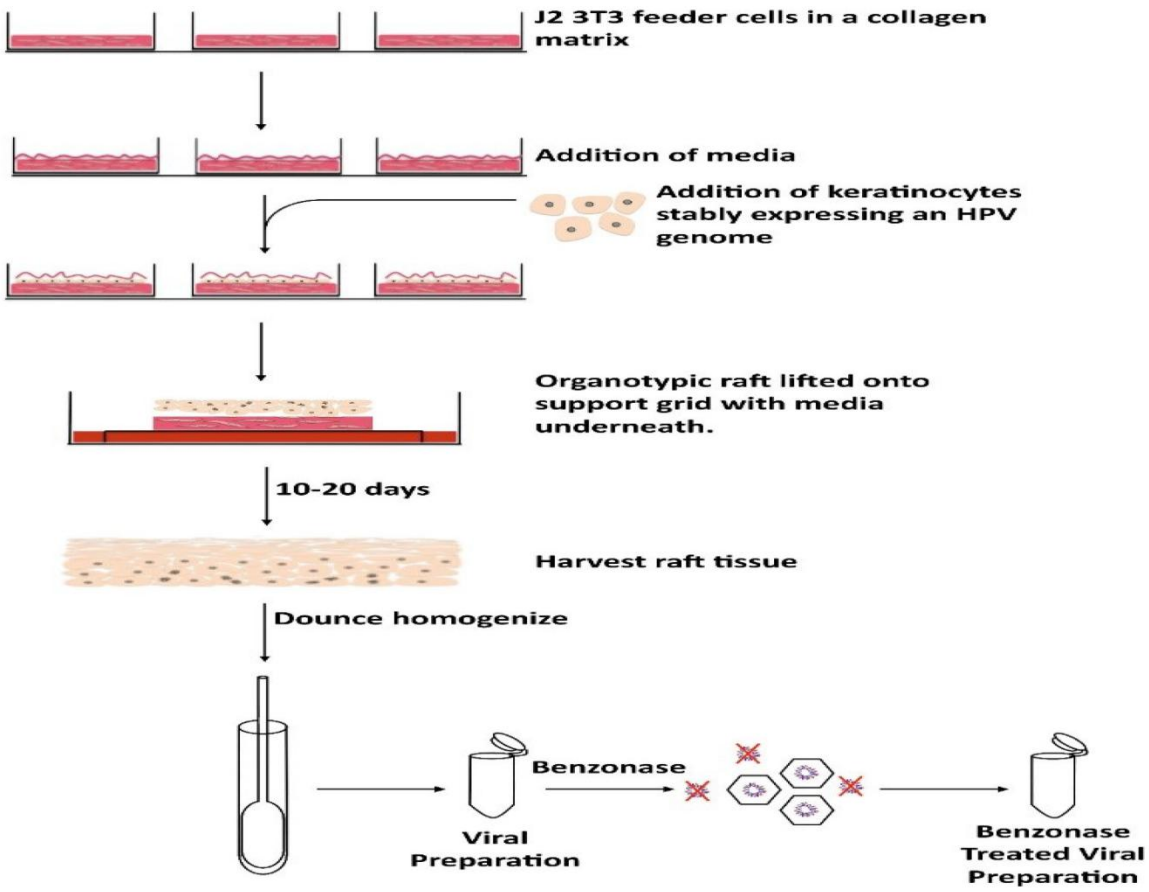

Figure 2.3 Organotypic raft culture system (adapted from Jennifer *et al* 2014). Diagrammatic representation of production of native HPV virion. Collagen plugs are developed by combining collegen and J2 3T3 feeder cells and then they are seeded by HPV-positive cells. When the cells are grown to confluence, the plugs are lifted onto a support grid. The rafts are then fed through media by diffusion under the grid. After 10 days and 20 days, the tissue is produced, homogenized, and benzonase treated to get a final viral preparation.

HPV Genomic organization & Life cycle

HPV virions consist of circular dsDNA, They are nonenveloped with icosahedral capsids. Genome size of viral HPV is approximately 8 kb in size. Inside the capsids, viral genomes develop linkage with cellular histone and forms chromatin like structure (Doorbar 2005; Larsen *et al* 1987). The HPV Viral genomes are made up of eight open reading frames (ORFs), all HPV types are produced from polycistronic mRNAs (Favre *et al* 1975; Zheng and Baker, 2006). These all eight HPV types ORFs can be subdivided into late and early genes, and an upstream regulatory region (URR) which is also called non-coding region (Fig. 2.4) (Buck *et al* 2008; Zheng and Baker, 2006).

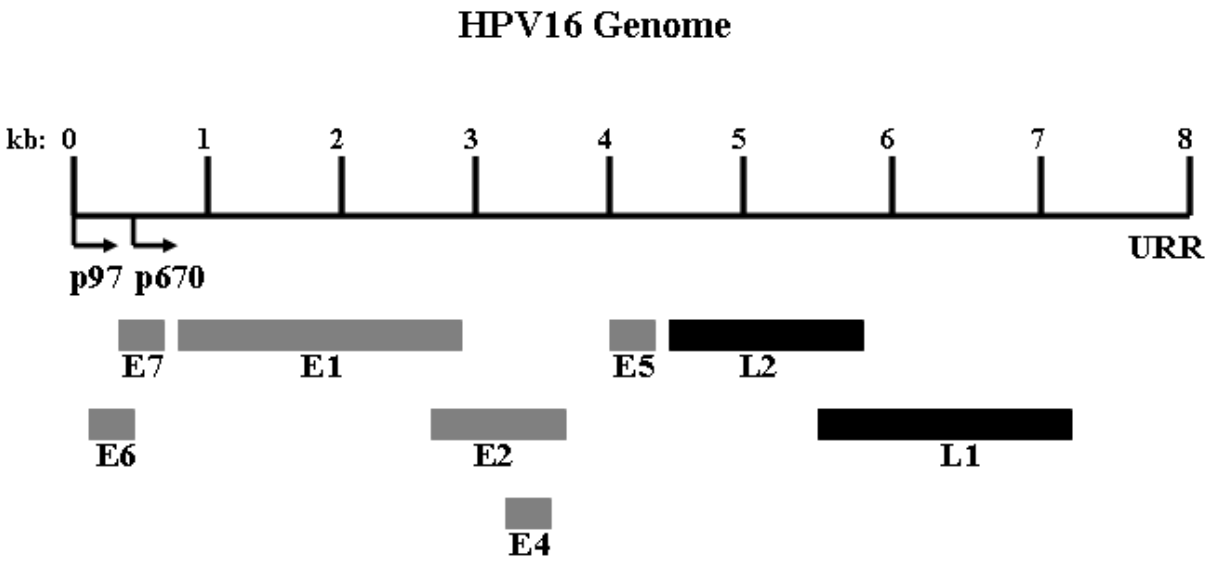

Figure 2.4 Linear Diagrammatic representation of HPV16 Genomic organization. Grey Bars indicate Early ORFs, and Black bars indicate late ORFs. Black arrows indicate early (p97) and late (p670) promoters. URR designate upstream regulatory region.

The early genes translate the E1, E2, E4 E5, E6, and E7 genes in to their respective proteins (Buck *et al* 2008; Zheng and Baker 2006; Doorbar *et al* 1986). E1 and E2 proteins are involved in regulation of viral replication and help in translation of other early genes (Conger *et al* 1989; Cripe *et al* 1987; Gloss and Bernard, 1990; Meyers *et al* 1992 and Mohr *et al* 1990). The spliced, E1^E4 protein could play role in disturbing the replication of viral genomes and restrain the transition of G2-M state. They also collapse the cellular keratin networks and thus helping mature virions to escape from cornified envelop (Brown *et al* 2006; Bryan and brown, 2000; Gambhira *et al* 2007; Knight *et al* 2006; Wang *et al* 2004; Wilson and laimins, 2005). Some early proteins for example E5, E6, and E7 are coded by the high-risk HPVs having oncogenic potential and they can transform the cell and help to stimulate the growth of host cells (Androphy *et al* 1987; Bedell *et al* 1991; Phelps *et al* 1988). The L1 and L2 proteins are encoded by late genes. L1 also called as the major capsid protein and L2 is called as the minor capsid protein (Buck *et al* 2008; Chen *et al* 2000; Doorbar and Gallimore,1987; Favre 1975).

Entry into Cell: The life cycle of HPV depend on the differentiation of host cell keratinocyte. Mature virions assemble only in terminally differentiated suprabasal cells (Barksdale and Bakers, 1993; Hummel *et al* 1992; Meyers *et al* 1992). Microabrasions of the epithelial tissue is considered the initial entry site for HPV virons through which HPV virions get enter in to the basal layer of cells (Belnap *et al* 1996). Basal layer cells are composed of stem cells and transit amplifying cells, and epithelial stem cells must be infected for a lesion to be maintained (Culp *et al* 2006; Doorbar 2005; Paterson *et al* 2005; Selinka *et al* 2003; Yoon *et al* 2001). Basal cells constantly divide and refill cells that are lost due to desquamation (Kirnbauer *et al* 1992). The HPV entry receptors are not confirmed, alpha integrin and heparin sulfate may be considered as two possible receptors (Bousarghin *et al* 2003; Day *et al* 2003; Fligge *et al* 2001;Hindmarsh and Laminis, 2007; Smith *et al* 2007). There is no detail available about the delivery of viral genome

into the nucleus; it is assumed that the N-terminus of L2 is cleaved within the endosome with the help of a cellular protease furin, then releasing a L2/genome complex into the cytosol (Bordeaux *et al* 2006; Fay *et al* 2004). The L2/genome complex within the cytosol interacts with syntaxin 18, which transport the complex to a perinuclear site (Smith *et al* 2007). L2 then moves with the genome into the nucleus through its NLS (Cumming *et al* 2004; Gloss and Bernard, 1990; Hirochika *et al* 1987; Kanaya *et al* 1997; Kyo *et al* 1995; Romanczuk *et al* 1990; Smith *et al* 2007).

Early genes: High-risk HPV transcription initiate by two major viral promoters (Conger *et al* 1999; Meyers *et al* 1992; Mohr *et al* 1990; Smith *et al* 2007). The early promoter starts from upstream of the E6 ORF and synthesizes transcripts that are referred as p97, in HPV31 it is p99, and in HPV18 it is p105 (Fig.2.4) (Mohr *et al* 1990).E1 and E2 are expressed first, and they assist in the establishment of 20 to 100 episomal copies per basal cell (Cripe *et al* 1987; Gloss and Bernard, 1990, Meyers *et al* 1992, Mohr *et al* 1990). E1 and E2 proteins form a complex with the origin of replication of virus and use cellular polymerases and some other necessary proteins to assist replication (Rolfe *et al* 1995).

E2 is a DNA binding protein that with the help of E1 protein regulates transcription of E6 and E7 from the early promoter (Dyson *et al* 1989; Neary, K., and D. DiMaio, 1989). The E6 and E7 proteins from high-risk HPV types are oncoproteins. High-risk E6 binds to the p53 a tumor suppressor protein. It forms a complex structure with the cellular ubiquitin ligase E6AP. The ubiquitin ligase activity of E6AP leads to the down regulation of p53 (Mc Phillips *et al* 2006; You *et al* 2004). High-risk E7 binds to different tumor suppressor's proteins such as the retinoblastoma (Rb) and cell cycle regulatory proteins, this leads to uncontrolled cellular replication (Bstein and McBride, 2000; Lehman and Botchan, 1998).

As basal cells with infected HPV divide, each daughter cell produce with a set of viral genome as viral genomes equally divide during mitosis (Fig. 2.5). After mitosis the one cell remains attached to the basal layer and the other cell migrate up through the suprabasal layers (Pang *et al* 1993). The cell begins a process of terminal differentiation during migration of the cell up through the strata (Fig. 2.2) (Morris *et al* 1993). In the case of normal epithelia (non-HPV-infected epithelia) cells normally leave the cell cycle when they separate from the basal layer (Fig. 2.5) (Banerjee *et al* 2006; Barksdale and Baker 1993; Bedell *et al* 1991; Swindle *et al* 1999). In the case of abnormal epithelia (HPV-infected epithelia) once cells detached they remain active mitotically because of the oncogenic properties of the E7 protein (Ozbun and Meyers 1998; Ozbun and Myers, 1999; Smith *et al* 2007).

Late genes: Initiation of late promoter takes place in a differentiation-dependent manner. Late genes activated when cells are grown in the host's stratifying/differentiating tissue or in case of *in vitro* through the use of organotypic culture techniques or methylcellulose. In HPV16 p670 is identified as late promoter (Barksdale and Baker, 1993). The purpose of the late promoter is to produce a transcript that translated in to L1 and L2 proteins (Fig. 2.4). E1^E4, and E5 are early proteins and both are translated in a differentiation-dependent manner. Both proteins may affect the replication of viral genomes. E1^E4 inhibit the G2-to-M transition and E5 affect by stimulation of cell cycle progression (Bird *et al* 2008; Buck *et al* 2004; Chromy *et al* 2006; chromy *et al* 2003). As, E1^E4, and E5 the L1 and L2 capsid proteins are expressed in a differentiation-dependent manner (Fig. 2.5) (Heino and Lambert, 2000, Holmgren *et al* 2005; Zhao *et al* 2000). It is clear from studies that when capsid proteins are expressed they assemble in to icosahedral structure with the help of chaperone proteins and proper redox environment (Bird *et al* 2008; Bordeaux *et al* 2006; Buck *et al* 2008; chromy *et al* 2006; chromy *et al* 2003; Doorbar and Gallimore, 1987; Fligge *et al* 2001; Heino and Lambert, 2000; Zhao *et al* 2000). It

is not known whether viral genome encapsidation takes place during capsid assembly or after capsid assembly but it is clear that L2 assist and E2 protein facilitate the encapsidation (Brown *et al* 2006; Bryan and Brown, 2000; Bryan and Brown, 2001).

Assembly and exit of virion: Capsid of papillomavirus are made up from a variety of viral components (L1, L2, viral DNA, and histones), in addition to viral component there are some cellular components that play a possible roles either directly or indirectly for the development or yield of capsid. (i.e. molecular chaperones, karyopherins, etc.) (Bryan and Brown, 2001).

For proper assembly of native virion some additional unknown viral or cellular factors are needed which helps in formation of correct disulfide bonds, proper cellular localization and initial interaction of capsid proteins and expression regulation of capsid protein. Mature virions escape from desquamated cornified cells with the help of E1^E4 proteins. These proteins interact with cellular keratin networks and collapse them allowing the virions to escape (Chen *et al* 2000; Hagensee *et al* 1994; Modis *et al* 2002).

The need for way out of virions from cornified cells is unclear, since individual virions within isolated, HPV11-infected cornified cells appear more infectious than individual virions purified away from cornified material (Fig. 2.5) (Sapp *et al* 1998; Buck *et al* 2005; Fligge *et al* 2001; Ishii *et al* 2005; Kondo *et al* 2007). It remains unclear what molecular interactions regulate the monomeric capsid proteins to assemble into complete structures during stratification and differentiation of the host cell keratinocyte.

In order to learn different aspects of the life cycle of HPV such as pathways of HPV infectivity, its immunogenicity, route of transmission and structure of virus, recent research mostly use recombinant papillomavirus particles such as (VLPs) virus-like particles, (QV) quasivirions a papillomavirus containg genome, (PsV) pseudovirions a papillomavirus based on gene transfer vectors, all these papillomavirus bypass the need for stratifying and differentiating tissue in the

production of native HPV. For recombinant papillomavirus particles in the perspective of stratifying and differentiating human epithelial tissue, we need to utilizing virions produced and derived from organotypic culture, xenograft, and/or native tissue.

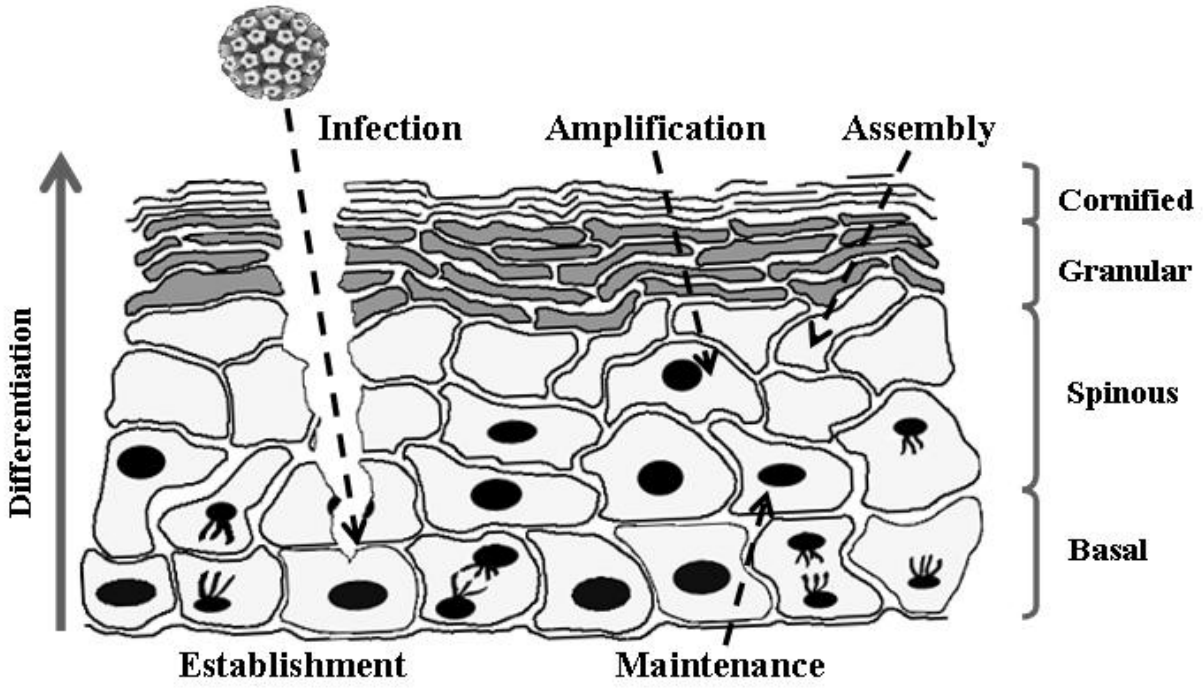

Figure 2.5 2D model of human epithelial tissue and HPV life cycle (Kondo *et al* 2007) **Microabrasions is the route for the entry of** HPV virions to establish an infection in basal epithelial cells. Within the tissue, viral genomes maintained 20-100 episomal copies per cell. Once entered, viral genomes travel up through the differentiating epithelial tissue as the mitotically active basal cells divide. Late gene expression and viral genome amplification takes place in suprabasal layers. Progeny virions escape with the envelope and permitting the viral life cycle to continue.

Capsids of papillomavirus

HPV icosahedral, non enveloped virions having diametre of approximately 50 to 60 nm (Trus *et al* 1997). Capsids of HPV are composed of 360 copies of L1 protein and an unknown amount of the L2 capsid protein (Buck *et al* 2008) L1 proteins assemble into pentamers through hydrophobic interactions (Kirnbauer *et al* 1992; Kirnbauer *et al* 1993). The 72 pentamers form the capsid. These pentamers are thought to be stabilized by a network of intra and inter-pentamerc disulphide interactions as well as hydrophobic interactions (Bossis *et al* 2005; Hagensee *et al* 1993; Kirnbauer *et al* 1993; Volper *et al* 1994). Inside the virion there is 8kb circular genome which is associated with histone and form chromatin like structure (Belnap *et al* 1996; Fligge *et al* 2001; Gambhira *et al* 2008). The capsid stability is increased by genome, which also increases resistance to environmental stresses such as proteolysis (Fligge *et al* 2001). It has been shown in previous studies that native HPV16 virus use a tissue-spanning redox gradient that help assembly and maturation events in the context of the complete papillomavirus life cycle (Conway *et al* 2009; Conway and Meyers, 2009; Conway *et al* 2011).

Biological role of Conserved L1 Cysteines Residues: Genetic and biochemical examination of human papillomavirus type 16 (HPV16) have shown that certain conserved L1 Cysteine residues are critical for capsid assembly, integrity and maturation. Since previous studies utilized HPV capsids produced in monolayer culture-based protein expression systems, the ascribed roles for these cysteine residues were not placed in the context of stratifying and differentiating human tissue the natural host environment for HPV. Previously function of few cysteine residues were analyzed by substitution of cysteine residues C175, C185, and C428 for serine which lead to a defect in the growth of endonuclease-resistant genomes and leads to the development of fragile mutant virions as compared to wild type virions. This data is similar to studies earlier done by

using virus-like particles (VLPs), recombinant HPV particles made in monolayer cell systems that also show an importance for C175, C185, and C428 in capsid stability (Ishii *et al* 2003).

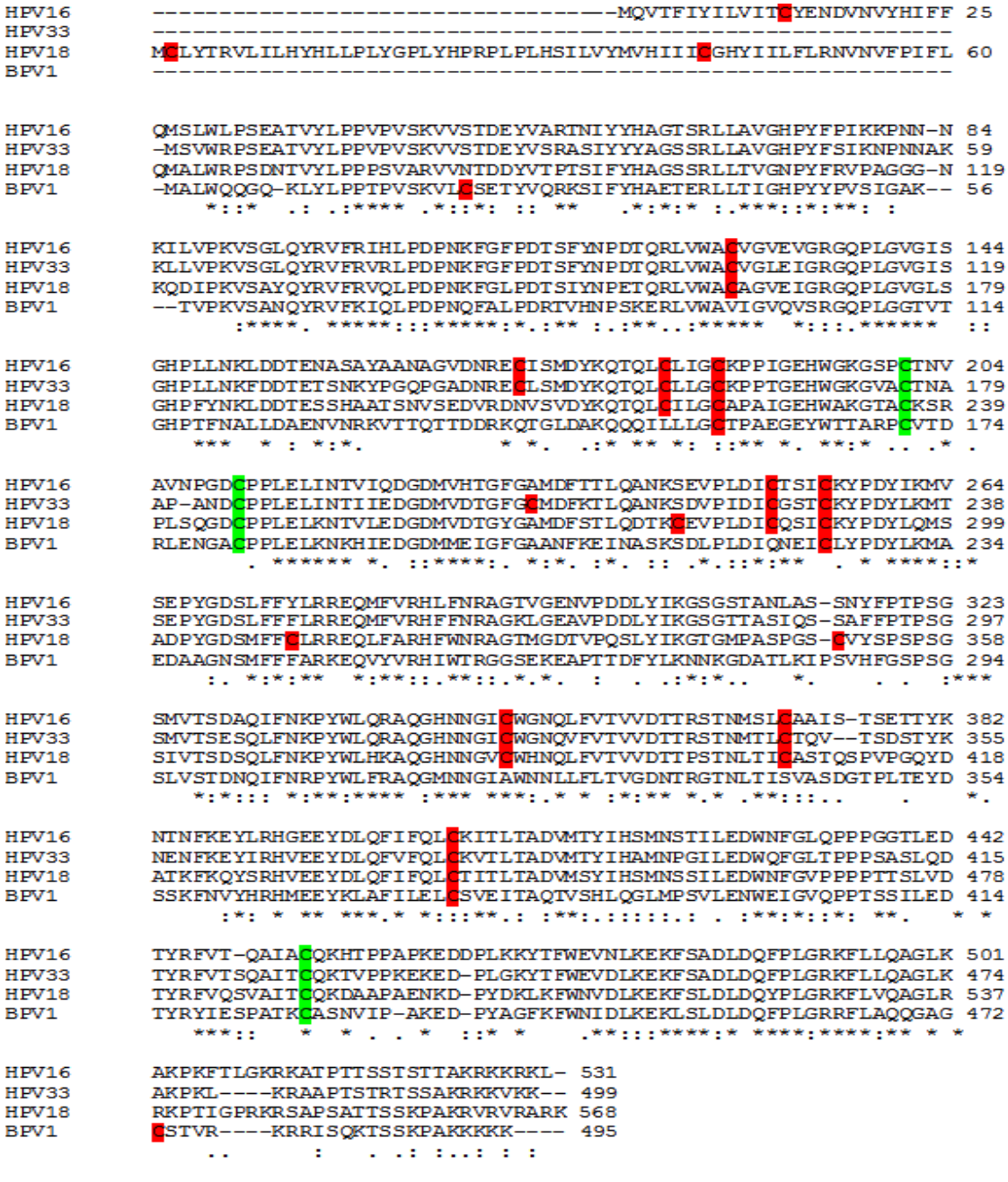

Figure 2.6 Genetic alignment of L1 amino acid sequences of papillomaviruses (Ishii *et al* 2003) Papillomavirus types are listed on the left. Cysteine residues in each protein sequence are highlighted. Cysteine residues are involve in inter-pentameric disulfide bond formation are highlighted in green whereas other cysteine residues are highlighted in red. Asterisks indicate 100% amino acid identity. Colons mean conservation of amino acid. Periods symbolize conservation of some of the amino acids. White space represents a lack of amino acid conservation.

Upstream Methionines and their role in L1 of HPVs

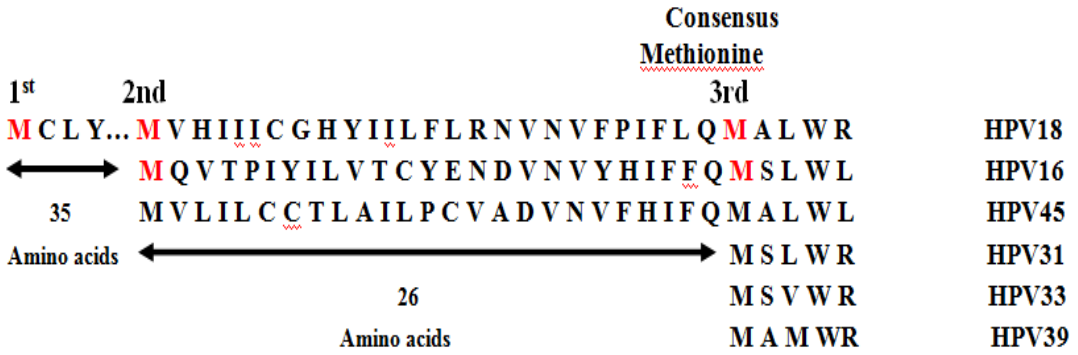

Figure 2.7 HPV Species L1 alignment showing upstream methionine and the consensus methionine

Historically a synthetic transfection system was used to generate virus like particles (VLP) for study expression vectors for the L1 capsid protein were constructed without the first in-frame (HPV16) or first and second in-frame methionines (HPV18). One of previous study done on sequences of major capsid protein of HPV16L1 shows that Major capsid contains two upstream initiation codons which would allow translation to begin at either nucleotide 5559 or 5637. However the formation of VLPs occur when the initiation codon at nucleotide 5637 is used for invitro expression studies. Sequence analysis of different HPV types associated with chemical outcome has showed that L1 sequence of cervical cancer associated viruses generally possess the ability to encode a longer translation product while the non cancer causing virus do not. The expression vectors begin the L1 open reading frame at a consensus methionine found when line up the N terminal L1 sequence of many HPV species (Elizabeth *et al* 2004).

Previously Lab has western blot evidence of wild type HPV16 L1 where two bands are visible. HPV16 has a methionine upstream of the consensus methionine and they suggest that it is possible that this provides the second form of L1.

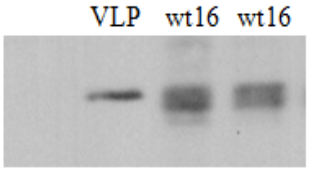

Figure 2.8 Western blot evidence of HPV16 L1 from Meyers Lab

Longer L1 protein could play a role in the development of cervical carcinoma and that HPVs with the potential to cause cervical cancer may be identified by the presence of an in-frame ATG situated 78 nucleotides upstream (Elizabeth *et al* 2004).

The Objective of the present study was to examine the prevalence and role of conserved cysteine and methionine mutants in HPV 16 L1.

1) Frequency and distribution of high-risk HPV types i.e. type 16 and type 18 in cervical cancer patients from twin cities of Pakistan and to check the association of HPV genotypes with cervical cancer among Pakistani females.
2) Role of Intra-pentameric three Cysteine Mutants (C161S, C229S, C379S) in HPV16 capsid formation.
3) Role of alternative or upstream Initiation Methionines in HPV16 capsid formation.

Chapter 3

MATERIALS AND METHODS

Prevalence and genotyping of high-risk human papillomavirus in women with cervical cancer in twin cities of Pakistan

3.1 Sample Collection:

Current study was designed and conducted at Atta-ur-Rahman School of Applied Biosciences (ASAB) National University of Sciences and Technology Islamabad (NUST) Pakistan. The study was approved by Ethics Committee of NUST. Around 67 formalin fixed paraffin-embedded (FFPE) cervical cancer biopsies reported from 2010 to 2012 were obtained from the government hospitals of Twin cities (Islamabad and Rawalpindi) including Pakistan Institute of Medical Sciences (PIMS), Holy family and Railway General Hospital Rawalpindi. Informed consent was obtained from patients included in the study. PIMS hospital is the one of biggest hospital of federal capital. The patients visiting the PIMS hospital are from various parts of the country especially from Punjab and Khyber Pakhtun Khwa (KPK) province and symbolize various ethnicities and broad social backgrounds. Biopsy samples were histopathologically confirmed by histopathologists as squamous cell carcinoma, adenocarcinoma, cervical intraepithelial neoplasia I (CINI), cervical intraepithelial neoplasia II (CINII) or cervical intraepithelial neoplasia III (CINIII) of the cervix.

3.2 DNA extraction & HPV detection through PCR

DNA was extracted from paraffin embedded tissue samples. 10-15μm thick sections of paraffin embedded cervical cancer tissue biopsy was deparaffinized with xylene. Rehydration in graded ethanol was carried out as described by Wright and Manos (Wright and Manos, 1990). The DNA was extracted by organic method and then extracted DNA was subjected to PCR. The consensus

sequence of HPV L1 gene was amplified by using previously reported sets of primers GP5+/GP6+ that can detect up to 44 different HPV genotypes (Table 3.1) (Baay et al 1996). The PCR conditions for these primers were as follows; for primers GP5/GP6, the total 25 ml PCR reaction mixture contained 100 ng -200 ng of template DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl2, 200 mM deoxynucleotide triphosphates (dNTPs), 0.2 pmol of each primer, and 0.2 U of Taq polymerase. The PCR thermal profile was: 95℃ for 5 min, and 35 cycles of 94℃ for 30 s, 45℃ for 30 s, 72℃ for 30 s, and final extension of 5 min at 72℃. PCR conditions for TS-16 and TS-18 primers were the same as for the GP primers, except that for TS-16 and TS-18 the annealing temperatures were 58℃ and 56℃ respectively. For beta globin primers, the PCR thermal profile was 94℃ for 5 min, and 35 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s, and 5 min final extension at 72℃.

Table 3.1: Oligonucleotide sequences used as primers for HPV Genotyping

Primers	Primer sequence (5'-)	Product size	Annealing Temperature
Beta globin	FP- ACACAACTGTGTTCACTAGC RP-CAACTTCATCCACGTTCACC	110bp	55°C
GP 5/ GP6	FP- TTTGTTACTGTGGTAGATAC RP- GAAAAATAAACTGTAAATCA	150bp	45°C
TS16	FP- GGTCGGTGGACCGGTCGATG RP- GCAATGTAGGTGTATCTCCA	96bp	58°C
TS18	FP- CCTTGGACGTAAATTTTTGG RP- CACGCACACGCTTGGCAGGT	115 bp	56°C

Beta globin for β-globin gene, GP5/GP6, general primers for HPV; TS16-F/TS16-R for HPV subtype 16 and TS18-F/TS-18R for HPV subtype 18. TS; type specific, FP; forward primer, RP; reverse primer

Role of intra-pentameric three cysteine's (C161, C229, C379) in biology of HPV16

3.3 Mutagenesis

pBSHPV16(114/B) DNA, a gift from M. Dürst, was used as the template for site-directed mutagenesis using Stratagene's Quikchange II XL Site-Directed Mutagenesis Kit as depicted already (Kirnbauer *et al* 1993). Clones of multiple mutant viral genome containing correct mutant sequences were isolated per each mutation and utilized in later experiments. Full length HPV16(114/B) genome with C379S substitution were created with the help of two complimentary oligonucleotides forward 5' CAGTTTATTTTTCAACTGTCTAAAATAACCTTAACTGCAG 3' and reverse 5' CTGCAGTTAAGGTTATTTTAGACAGTTGAAAAATAAACTG 3', change of GC to CT at nucleotides number 6772-6773. Similarly to create a genome of full-length HPV16(114/B) with a C161S substitution, the two complimentary oligonucleotides forward 5' GTGTTTAATTGGTTCTAAACCACCTATAGGGGAACAC 3' and reverse 5' GTGTTCCCCTATAGGTGGTTTAGAACCAATTAAACAC 3', with changed GC to CT at nucleotides 6119-6120 were used. In order to generate third full-length HPV16(114/B) genome with a C229S substitution, the two complimentary oligonucleotides forward 5' GATATTTGTACATCTATTTCTAAATATCCAG 3' and reverse 5' CTGGATATTTAGAAATAGATGTACAAATATC 3', changed GC to CT at nucleotides 6323-6324 were used. In order to generate these mutants, the most common serine codon TCT was used in the HPV16 (114/B) L1 ORF.

3.4 Keratinocyte cultures and electroporation

HFKs were detached and developed from infant circumcision as illustrated already (McLaughlin *et al* 2003). For electroporations, 30 µg of the mutated DNA of virus was digested with the help of BamHI, this results in linearizing the viral DNA and isolating it from the vector sequence. HFKs were electroporated with the prepared DNA as described previously (Meyers *et al* 1992; McLaughlin *et al* 2003). Different cell lines were obtained for each mutated genome. The mutated section of the viral genome was confirmed by sequencing for each cell line used.

3.5 Southern blot hybridization

Entire cellular DNA was extracted as described earlier (McLaughlin *et al* 2003). 5 µg of complete cellular DNA was digested with BamHI for HPV16 genome linearization.0.8% agrose gel electrophoresis were used to separate samples and transferred onto a GeneScreen Plus membrane (New England Nuclear Research Products) as previously described (Ozbun and Meyers, 1998). Hybridization of the membrane used an HPV16-specific, whole-genome probe as previously described (McLaughlin *et al* 2003).

3.6 Organotypic raft culture-derived virion production

Immortalized HFK lines contaning episomal HPV16 DNA, were grown in monolayer culture with E-medium in the presence of mitomycin C-treated J2 3T3 feeder cells (McLaughlin et al 2003). Raft tissues were grown for 20 days as described earlier (Conway *et al* 2009; McLaughlin *et al* 2003).

3.7 Histology

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) as previously described (Meyers *et al* 1992).

3.8 HPV isolation

For Optiprep (Sigma-Aldrich) fractionation, quantitative RT-PCR (RT-qPCR) and qPCR-based DNA titering assays three rafts were prepared by dounce homogenization in 500 µl of phosphate buffer (0.05 M sodium phosphate [pH 8.0], 2 mM $MgCl_2$). Homogenizers were rinsed with 250 µl of phosphate buffer. Then, 1.5 µl (375 U) of benzonase was added to 750 µl of virus prep, followed by incubation at 37°C for 1 hour. Samples were brought to 1 M NaCl by adding 195 µl of 5 M NaCl. Then, samples were mixed and centrifuged at 4°C for 10 minutes at 10,500 rpm in a microcentrifuge. The supernatants were stored at -20°C.

3.9 Optiprep purification of virions

Optiprep purification was performed as described previously (Conway *et al* 2009; Buck *et al* 2007). Briefly, 27%, 33%, and 39% Optiprep gradients were produced by underlayering. Gradients were allowed to diffuse for 1 hour at room temperature. Then, approximately 350 µl of benzonase-treated virus prep was layered on top of the gradient. Tubes were centrifuged in a SW55 rotor (Beckman) at 234,000 X *g* for 3.5 hours at 16°C. Post centrifugation, eleven, 500 µl fractions were carefully collected starting from the top of each tube (top=1 and bottom=11).

3.10 qPCR-based DNA titering assay

To detect endonuclease-resistant genomes in viral preps or Optiprep fractions, only benzonase-treated viral preps were utilized so that all non-encapsidated genomes were digested (Conway *et al* 2009; Conway *et al* 2009; Conwy *et al* 2011). To release all encapsidated viral genomes, 10 µl

of viral prep or Optiprep fraction was added to 2 µl of proteinase K, 10 µl of 10% sodium dodecyl sulfate, and brought up to 200 µl with Hirt buffer. Tubes were rotated at 37°C for 2 hours. Immediately, an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was extracted for the aqueous phase. An equal amount of chloroform was added and again extracted for the aqueous phase. DNA was ethanol precipitated overnight at -20°C. After centrifugation, the DNA pellet was washed with 70% ethanol and resuspended in 20 µl of Tris-EDTA overnight. To detect viral genomes, a SYBR green PCR kit (Bio-Rad) was utilized. Amplification of the viral target was performed in 0.2 ml, 96-well PCR plates (Bio-Rad) with a total reaction volume of 25 µl. Then, 1 µl of each endonuclease-resistant viral genome prep was analyzed in duplicate for each independent experiment. Amplification of HPV16 genomes was performed using primers complimentary to the HPV16 E2 gene: forward 5' CCATATAGACTATTGGAAACACATGCGCC3' and reverse 5' CGTTAGTTGCAGTTCAATTGCTTGTAATGC 3'. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). A standard curve was generated by amplifying 1 µl aliquots of 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 serially diluted pBSHPV16 copy number controls. Acceptable R^2 values for standard curves were at or above 0.99. A Bio-Rad CFX-96 Real-Time qPCR machine and software were utilized for PCR amplifications and subsequent data analysis. The PCR thermocycling profile was as follows: a 15-minute hot-start at 95°C, followed by 40 cycles at 15 seconds at 94°C, 30 seconds at 52°C, and 30 seconds at 72°C. The data was collected during the extension phase.

3.11 Infectivity assays

In Dulbecco modified Eagle medium 10% fetal bovine serum were added and in this mixture HaCaT cells were grown. These cells were seeded in50,000 cells/well in 24-well plates, along

with 2 mM glutamine, 1 mM pyruvate, 100 U of penicillin/ml, and 100 µg of streptomycin/ml (Conway *et al* 2009; Conway *et al* 2009; Conwy *et al* 2011). Total volume of 0.5 ml cell culture medium were added in viral preps of HPV16. Medium was removed from HaCaT cells, and viral prep of 0.5 ml was added per well. To develop infection a multiplicity of infection (MOI) of 5 for non-fractionated virus was used. The cells incubation time with virus were 48 hours at 37°C. mRNA was harvested with the RNeasy kit (Qiagen). Amplification of viral target and endogenous cellular control target was done by, 96-well PCR plates (Bio-Rad). All reactions were done in duplicate especially containing RNAs from virus-infected cells. RT-qPCR was performed by using the Quantitect probe RT-PCR kit (Qiagen). The HPV16 E1^E4 primers used were: forward 5' GCTGATCCTGCAAGCAACGAAGTATC 3' and reverse 5' TTCTTCGGTGCCCAAGGC 3' these primers were used in concentrations of 4 µM. A fluorogenic, probe of HPV16 E1^E4which were dual-labeled (5'-6-FAM-CCCGCCGCGACCCATACCAAAGCC-BHQ-1-3') was used at a final concentration of 0.2 µM to detect E1^E4 cDNA. As an internal control TATA-binding protein (TBP) was amplified by utilizing the primers: 5'-CACGGCACTGATTTTCAGTTCT-3' and 5'-TTCTTGCTGCCAGTCTGGACT-3'. A fluorogenic, TBP probe which were dual labled (5'-5-HEX-TGTGCACAGGAGCCAAGAGTGAAGA-BHQ-1-3') was used at a final concentration of 0.3 µM to detect TBP cDNA. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). RT-qPCRs were done by using the CFX-96 (Bio-Rad).

3.12 Western blot analysis

Aliquots from organotypic tissue extracts were boiled for 10 minutes in presence of 2 mercaptoethanol (2-ME) loading buffer and after that they were loaded onto a 7.5% polyacrylamide gel. HPV16 L1 was detected by using the anti-HPV16 L1 monoclonal antibody

Camvir-1 (BD Pharmigen) which was utilized at a 1:2,000 dilution. An HRP-linked sheep anti-mouse secondary antibody was utilized at a 1:8,000 dilution. Membranes were washed with PBST after the addition of each antibody. All antibodies were diluted in Starting Block (Thermo Scientific). HRP was detected using an ECL kit (Perkin Elmer).

Role of alternative or upstream Initiation Methionines in biology of HPV16L1

3.13 DNA reagents

pBSHPV16 (114/B) DNA, was used as template for site-directed mutagenesis. Stratagene's Quikchange II XL Site97 Directed Mutagenesis Kit was utilized for mutagenesis. The PCR were used with a high-fidelity DNA polymerase, *PfuUltra*, and digeston were performed by subsequent *DpnI* digestion. *DpnI* is an endonuclease, with target sequence 5´-Gm6ATC-3 that is specific for methylated and hemimethylated DNA. *DpnI* digests DNA templates that originated from *dam* methylating *E. coli,* which facilitates the purification of non-methylayted PCR amplimers that contain the desired mutation. Following transformation of XL10 Ultracompetent *E. coli* cells and selection of the mutant amplimers (Stratagene, La Jolla, Calif.), we screened out products resulting from random mutations generated from PCR amplification by extracting plasmid DNA from many isolated bacterial clones and sequencing the complete L1 ORF to identify clones with desired nucleotide substitutions and a wild type sequence background. For each mutation, multiple clones with the correct sequences were isolated and utilized in subsequent experimentation. To create a full length, HPV16(114/B) genome with a C175S substitution, the two complimentary oligonucleotides, forward 5'CTGGGGCAAAGGATCCCCATCTACCAATGTTGCAGTAAATC3' and reverse 5'GATTTACTGCAACATTGGTAGATGGGGATCCTTTGCCCCAG3', changed G to C at nucleotide 6161. To create a full-length, HPV16 (114/B) genome with a C185S substitution, the two complimentary oligonucleotides, forward

5’GCAGTAAATCCAGGTGATTCTCCACCATTAGAG3’ and reverse 5’CTCTAATGGTGGAGATCACCTGGATTTACTGC3’, changed G to C at nucleotide 6191. To create a full-length, HPV16 (114/B) genome with a C428S substitution, the two complimentary oligonucleotides, forward 5’GTAACCCAGGCAATTGCTTCTCAAAAACATACACCTCC3’ and reverse 5’GGAGGTGTATGTTTTTGAGAAGCAATTGCCTGGGTTAC3’, changed G to C at nucleotide 6917. To create a full-length, HPV16 (114/B) genome with C175,185S substitutions, the two complimentary oligonucleotides, forward 5’GCAGTAAATCCAGGTGATTCTCCACCATTAGAG3’ and reverse 5’CTCTAATGGTGGAGATCACCTGGATTTACTGC3’, changed of G to C at nucleotides 6161 and 6191, respectively. The C175, 185S amplimers were made from the C175S template. In all mutations serine codon TCT, was introduced.

3.14 Native virion production

Episomal HPV16DNA were maintained in immortalized HFK lines these episomes further were were grown in monolayer culture contaning E medium in the presence of mitomycin C-treated J2 3T3 feeder cells. Raft tissues were grown as described earlier.

3.15 Keratinocyte cultures, and electroporation

HFKs were detached and developed from infant circumcision as illustrated already (McLaughlin *et al* 2003). For electroporations, 30 µg of the mutated DNA of virus was digested with the help of BamHI, this result in linearizing the viral DNA and isolating it from the vector sequence. HFKs were electroporated with the prepared DNA as described previously (Meyers *et al* 1992; McLaughlin *et al* 2003). Different cell lines were obtained for each mutated genome. The mutation section of the viral genome was confirmed by sequencing for each cell line used.

3.16 Southern blot hybridization

Entire cellular DNA was extracted as described earlier (McLaughlin *et al* 2003). 5 µg of complete cellular DNA was digested with BamHI in order to linearize HPV16 genome. 0.8% agrose gel electrophoresis were used to separate samples and transferred onto a GeneScreen Plus membrane (New England Nuclear Research Products) as previously described. Hybridization of the membrane used an HPV16-specific, whole-genome probe as previously described.

3.17 Histology and immunohistochemical staining.

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) as previously described (Meyers *et al* 1992). For staning of Sections anti-HPV16 L1 monoclonal antibody, Camvir-1 (BD Pharmingen), diluted 1:5,000 in PBS were used. For Immunostaining Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) were used according to the manufacturer's instructions.

3.18 HPV isolation

For Optiprep (Sigma-Aldrich) fractionation, quantitative RT-PCR (RT-qPCR) and qPCR-based DNA titering assays three rafts were prepared by dounce homogenization in 500 µl of phosphate buffer (0.05 M sodium phosphate [pH 8.0], 2 mM $MgCl_2$). Homogenizers were rinsed with 250 µl of phosphate buffer. Then, 1.5 µl (375 U) of benzonase was added to 750 µl of virus prep, followed by incubation at 37°C for 1 hour. Samples were brought to 1 M NaCl by adding 195 µl of 5 M NaCl. Then, samples were mixed and centrifuged at 4°C for 10 minutes at 10,500 rpm in a microcentrifuge. The supernatants were stored at -20°C.

3.19 Optiprep purification of virions

Optiprep purification was performed as described previously (Conway *et al* 2009; Buck *et al* 2007). Briefly, 27%, 33%, and 39% Optiprep gradients were produced by underlayering. Gradients were allowed to diffuse for 1 hour at room temperature. Then, approximately 600 µl of benzonase-treated virus prep was layered on top of the gradient. Tubes were centrifuged in a SW55 rotor (Beckman) at 234,000 X *g* for 3.5 hours at 16°C. Post centrifugation, 11, 500 µl fractions were carefully collected starting from the top of each tube (top=1 and bottom=11).

3.20 Quantitative RT-qPCR infectivity assays

In Dulbecco modified Eagle medium 10% fetal bovine serum were added and in this mixture HaCaT cells were grown. These cells were seeded in50,000 cells/well in 24-well plates, along with 2 mM glutamine, 1 mM pyruvate, 100 U of penicillin/ml, and 100 µg of streptomycin/ml (Conway *et al* 2009;Conway *et al* 2009; Conwy *et al* 2011). Total volume of 0.5 ml cell culture medium were added in viral preps of HPV16. Medium was removed from HaCaT cells, and viral prep of 0.5 ml was added per well. To develop infection an MOI of 5 for non-fractionated virus was used. The cells incubation time with virus were 48 hours at 37°C. mRNA was harvested with the RNeasy kit (Qiagen). Amplification of viral target and endogenous cellular control target was done by, 96-well PCR plates (Bio-Rad). All reactions were done in duplicate especially containing RNAs from virus-infected cells. RT-qPCR was performed by using the Quantitect probe RT-PCR kit (Qiagen). The HPV16 E1^E4 primers used were: forward 5’ GCTGATCCTGCAAGCAACGAAGTATC 3’ and reverse 5’ TTCTTCGGTGCCCAAGGC 3’ these primers were used in concentrations of 4 µM. A fluorogenic, probe of HPV16 E1^E4which were dual-labeled (5’-6-FAM-CCCGCCGCGACCCATACCAAAGCC-BHQ-1-3’) was used at a final concentration of 0.2 µM to detect E1^E4 cDNA. As an internal control TATA-binding

protein (TBP) was amplified by utilizing the primers: 5'-CACGGCACTGATTTTCAGTTCT-3' and 5'-TTCTTGCTGCCAGTCTGGACT-3'. A fluorogenic, TBP probe which were dual labled (5'-5-HEX-TGTGCACAGGAGCCAAGAGTGAAGA-BHQ-1-3') was used at a final concentration of 0.3 µM to detect TBP cDNA. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). RT-qPCRs were done by using the CFX-96 (Bio-Rad).

3.21 qPCR-based DNA encapsidation assay

To detect endonuclease-resistant genomes in viral preps or Optiprep fractions, only benzonase-treated viral preps were utilized so that all non-encapsidated genomes were digested (Conway *et al* 2009;Conway *et al* 2009; Conwy *et al* 2011). To release all encapsidated viral genomes, 10 µl of viral prep or Optiprep fraction was added to 2 µl of proteinase K, 10 µl of 10% sodium dodecyl sulfate, and brought up to 200 µl with Hirt buffer. Tubes were rotated at 37°C for 2 hours. Immediately, an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) was added and the mixture was extracted for the aqueous phase. An equal amount of chloroform was added and again extracted for the aqueous phase. DNA was ethanol precipitated overnight at -20°C. After centrifugation, the DNA pellet was washed with 70% ethanol and resuspended in 20 µl of Tris-EDTA overnight. To detect viral genomes, a SYBR green PCR kit (Bio-Rad) was utilized. Amplification of the viral target was performed in 0.2 ml, 96-well PCR plates (Bio-Rad) with a total reaction volume of 25 µl. Then, 1 µl of each endonuclease-resistant viral genome prep was analyzed in duplicate for each independent experiment. Amplification of HPV16 genomes was performed using primers complimentary to the HPV16 E2 gene: forward 5' CCATATAGACTATTGGAAACACATGCGCC3' and reverse 5' CGTTAGTTGCAGTTCAATTGCTTGTAATGC 3'. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). A standard curve was generated by amplifying 1

µl aliquots of 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 serially diluted pBSHPV16 copy number controls. Acceptable R^2 values for standard curves were at or above 0.99. A Bio-Rad CFX-96 Real-Time qPCR machine and software were utilized for PCR amplifications and subsequent data analysis. The PCR thermocycling profile was as follows: a 15-minute hot-start at 95°C, followed by 40 cycles at 15 seconds at 94°C, 30 seconds at 52°C, and 30 seconds at 72°C. The data was collected during the extension phase.

3.22 Immunoblot analysis

Aliquots from organotypic tissue extracts were boiled for 10 minutes in presence of 2 mercaptoethanol (2-ME) loading buffer and after that they were loaded onto a 7.5% polyacrylamide gel. HPV16 L1 was detected by using the anti-HPV16 L1 monoclonal antibody Camvir-1 (BD Pharmigen) which was utilized at a 1:4,000 dilution.

Chapter 4

RESULTS

In almost all cases of cervical cancer, Human papillomavirus (HPV) is the etiological agent for the development of cervical cancer (Walbommers *et al.,* 1999). There is very strong association between cancer and high risk HPV types 16, 18, 31 and 45 (de Villiers *et al.,* 2004; Longworth *et al.,* 2004). Cancer of cervix is one of the major causes of death among women in developing countries (de Sanjose *et al*., 2010). Pakistan is an underdeveloped country and screening of HPV is usually not implemented. In Pakistan there is lack of data in relation to HPV related cervical cancer among female patients in various parts of the country including, twin cities (Rawalpindi & Islamabad) of Pakistan. Therefore, one of the objectives was to explore the prevalence of HPV and its high risk types among women of twin cities of Pakistan. Furthermore, it was intended to explore the role of some conserved moieties in the biology of the major capsid protein of the most prevalent HPV subtype in local Cervical cancer patients.

4.1 Detection and genotyping of HPV in FFPE cervical cancer samples

DNA was isolated from 67 tissue samples (FFPE) of cervical cancer patients. For genotyping β-globin gene was used as positive control. All the samples were first subjected to HPV DNA detection using consensus primers based on conserved region of L1 gene. GP5+/GP+6 primer pair was used to amplify 150 bp region in the L1 gene which was detected on 2% Agarose gel (Figure 4.1). Out of 67 formalin-fixed paraffin-embedded cervical cancer samples 59 were HPV positive.

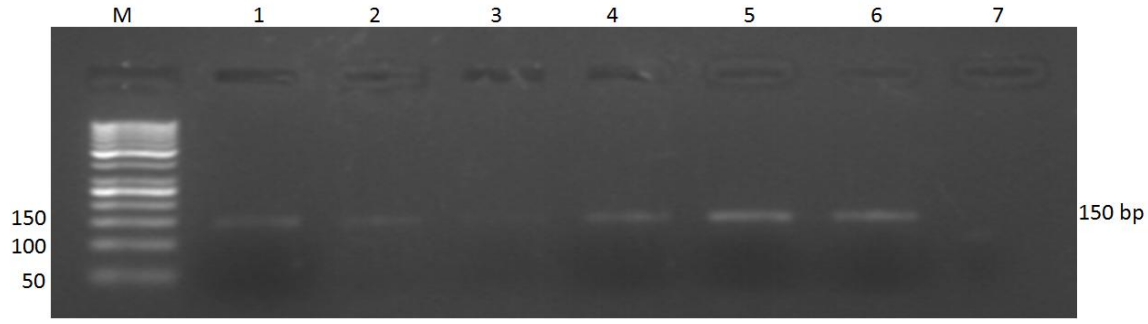

Figure 4.1: PCR-based detection of HPV-DNA in cervical lesions. 2% Agarose gel showing 150bp long PCR product obtained by amplification of a region in L1 gene in HPV DNA by using GP5+/GP6+ consensus primers. Lane M shows 50bp ladder. Sample numbers 1-5 are positive for HPV; lanes 6 and 7 show positive and negative control respectively.

Furthermore, the samples were genotyped for HPV high risk type 16 and 18 using TS16 and TS18 primer pairs already described. The PCR product of 96bp represents HPV type 16 and 115bp represents type18 (Figure 4.2 & 4.3).

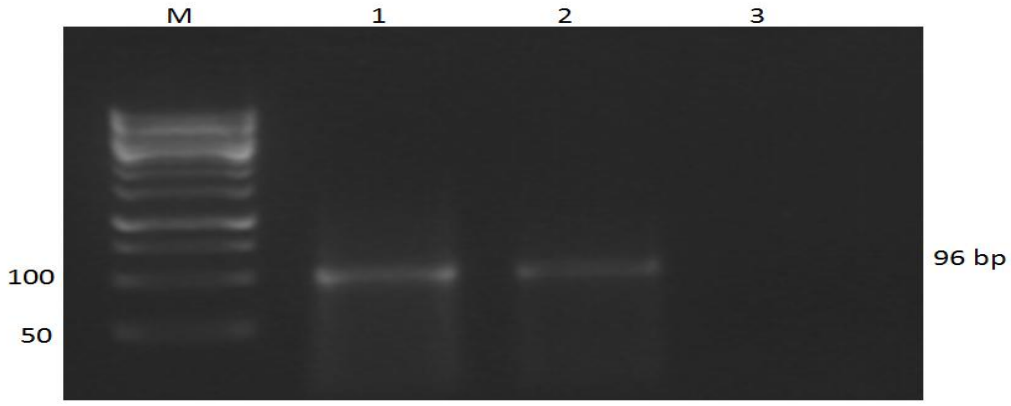

Figure 4.2 PCR-based detection of HPV16-DNA in cervical lesions. 2% Agrose gel showing 96bp long PCR product obtained by amplification of a gene in HPV16-DNA by using HPV16 type specific primers. Lane M shows 50bp ladder. Sample number1 is positive for HPV16; lanes 2 and3 show positive and negative control respectively.

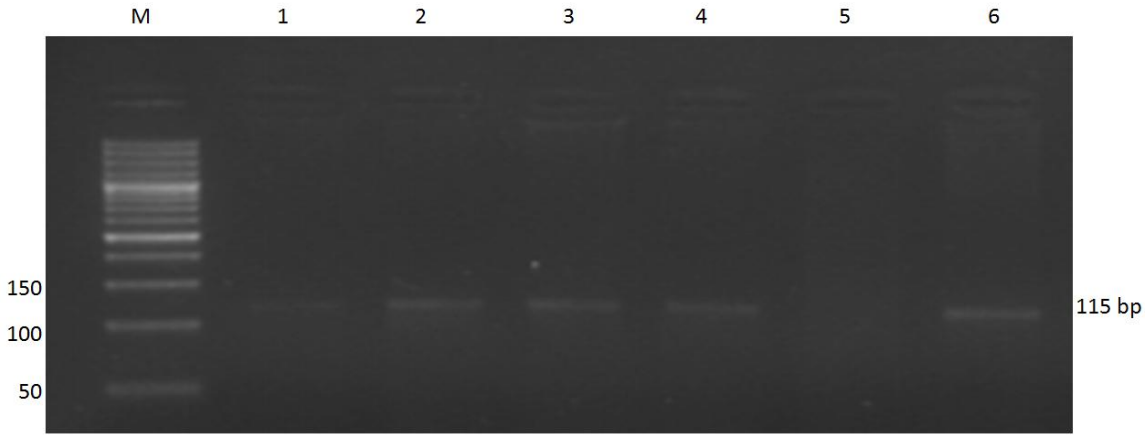

Figure 4.3 PCR-based detection of HPV18-DNA in cervical lesions. 2% Agarose gel showing 115bp long PCR product obtained by amplification of a region in L1 gene in HPV18- DNA by using HPV18 consensus primers. Lane M shows 50bp ladder. Sample numbers 1-4 are positive for HPV18; lanes 5 and 6 show negative and positive control respectively.

The paraffin embedded biopsies were screened for high risk HPV type 16 and 18 with the help of PCR. HPV type 16 detected in 30 (44.8%) cervical cancer biopsies, HPV-18 was found in 22 (32.8%) of cervical specimens, while 7 (10.4%) of cervical specimens were positive for HPV but negative for type 16 or 18 and 8(11.9%) cervical cancer biopsies were negative for HPV primers. Figure 4.4 represents the Prevalence of HPV genotypes among women of twin cities Pakistan.

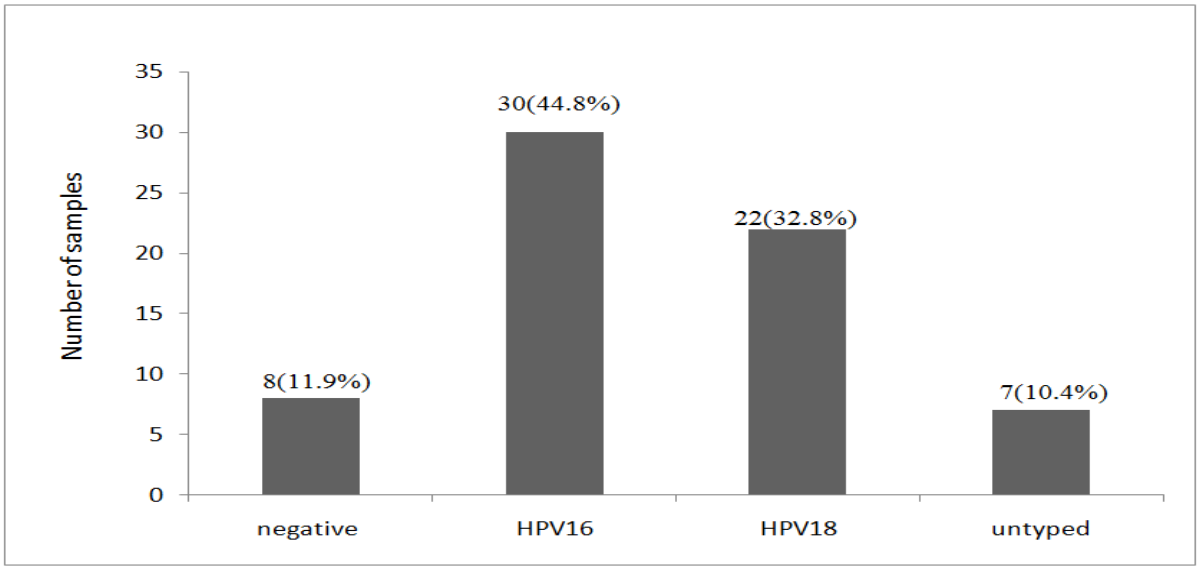

Figure 4.4 Distribution of HPV types 16 and 18 in cervical lesions. Fifty nine out of 67 formalin-fixed paraffin-embedded cervical cancer samples were HPV Positive. HPV types 16 was detected in 44.8% (30) of cervical cancer biopsies, and 32.8% (22) of cervical specimens were positive for HPV18. 10.4% (7) samples were HPV positive but the HPV type could not be determined whereas in 11.9% (8) cervical cancer biopsies HPV could not be detected through GP5+/GP6+ PCR.

The incidence of the HPV according to histopathological diagnosis (Table 4.1) showed that highest prevalence of HPV infection was found in SCC samples then in adenocarcinomas samples and Low percentage of infection was observed in CINI, CINII and CINIII samples. HPV 16 was detected in pre-cancerous lesions.

Table 4.1: HPV 16 and 18 genotype distribution in paraffin embedded cervical samples

Histo Pathological Status	HPV genotypes			Total HPV positive
	16	18	untyped HPV	
SCC	14 (42.42%)	15 (45.45%)	4 (12.12%)	33
Adenocarcinomas	9 (50%)	7 (38.88%)	2 (11.11%)	18
CIN1	-	-	1 (100%)	1
CIN2	1 (100%)	-	-	1
CIN3	6 (100%)	-	-	6
Total	30	22	7	59

SCC; Squamous cell carcinoma, CIN; Cervical intraepithelial neoplasia

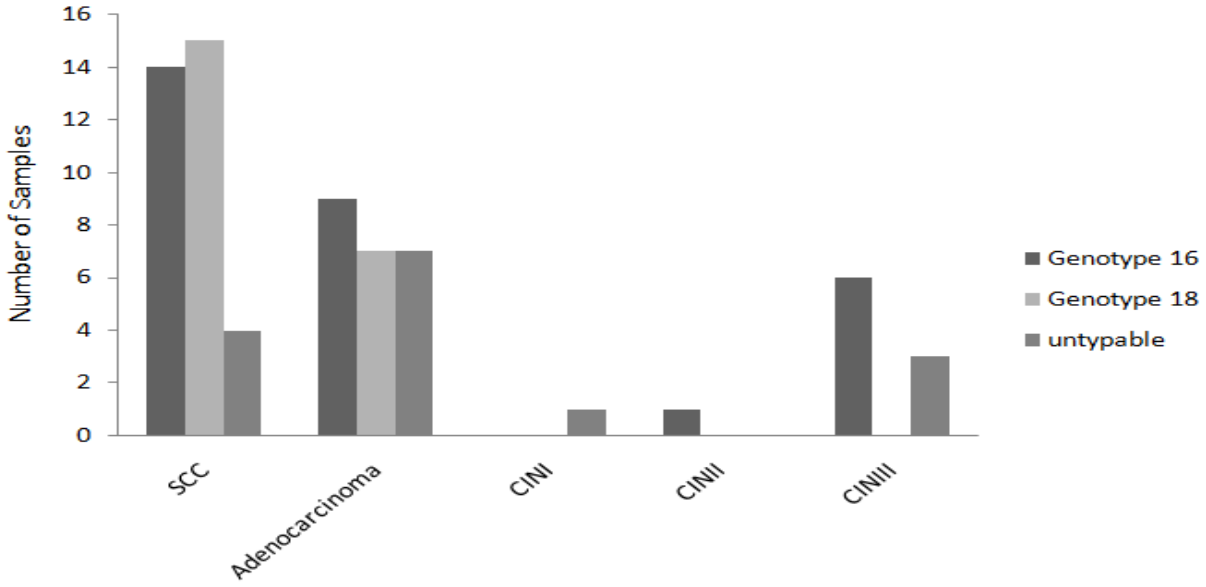

Figure 4.5 Graphical representation of HPV genotype 16 and 18 distributions in paraffin-embedded cervical samples. The rate of SCC was higher and associated with HPV18, the incidence of CINI, CINII were low and incidence of Adenocarcinoma and CINIII has moderate and were associated with incidence of HPV16.

The Distribution of HPV in different age groups are shown in Table 4.2. The HPV genotype distribution by age demonstrated that the age of 41-60 years had highest prevalence of HPV and HPV 16 and HPV 18 were found in all age range of patients with abnormal cervical biopsies. Graphic representations of HPV genotype according to age group of patients are shown in Figure 4.6.

Table 4.2: Distribution of HPV genotype 16 and 18 in different age groups

Age Group	HPV genotype			Total HPV positive
	16	18	untyped HPV	
21-30	1(50%)	-	1(50%)	2
31-40	4 (57.14%)	2 (28.57%)	1(14.28%)	7
41-50	7 (50%)	5 (35.71%)	2 (14.28%)	14
51-60	12 (63.15%)	7 (36.84%)	-	19
61-70	2 (25%)	3 (37.5%)	3 (37.5%)	8
71-80	1 (100%)	-	-	1
Unknown	3 (37.5%)	5 (62.5%)	-	8
Total	30	22	7	59

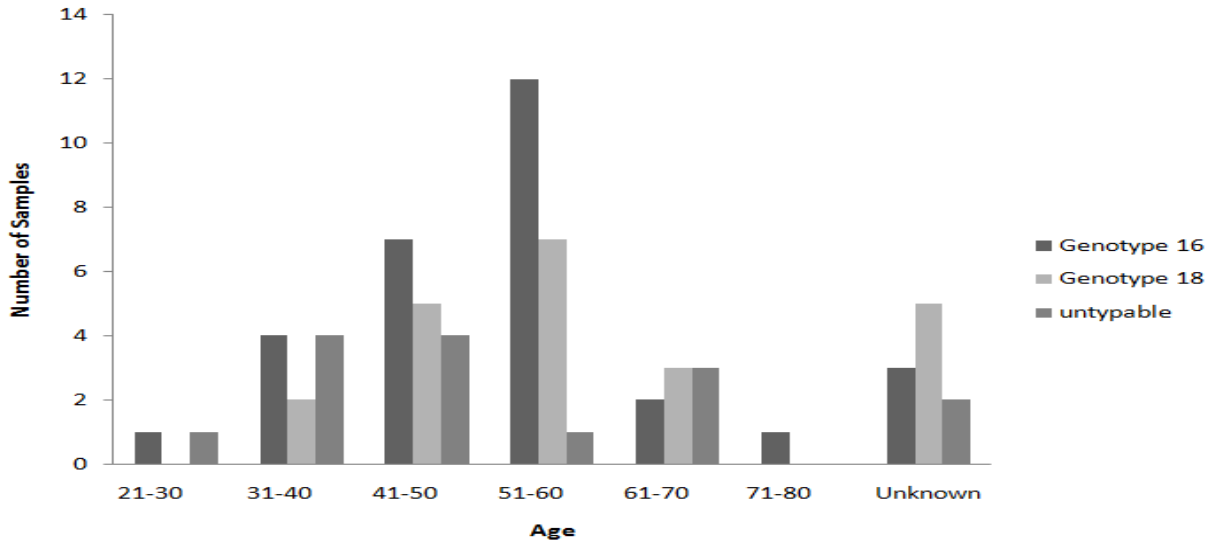

Figure 4.6: Graphical representation of HPV genotype 16 and 18 distribution among various age group. The maximum rate of cervical cancer was found in age group 51-60 followed by the 41-50 and 31-40 age groups respectively. The rate of HPV16 & HPV18 were higher in age group 51-60.

4.2: Role of Conserved Cysteine's in HPV16 Encapsidation

Establishment of HPV16 L1 C161S, C229S, and C379S cell lines

For the development of producer cell lines primary human foreskin keratinocytes (HFKs) were electroporated with wild-type and site-directed mutagenized HPV16 mutants which were already linearized. The objective was to make native virions from natural environment i.e from differentiating epithelia. Southern blots were performed to confirm the maintenance and recircularization of episomal HPV16 viral genomes in cell lines of HPV16 L1 C161S, C299S and C379S (data not shown)

Multiple episomal DNA-containing cell lines were produced for each mutant genome and checked in case any other sequence changes were introduced outside of L1 during the mutagenesis protocol. Open Reading frame of L1 were sequenced to confirm that the intended mutation was present, and that no other L1 mutations were introduced.

After the confirmation that cell lines have episomal viral DNA, they were grown in organotypic culture. 10 and 20 days Organotypic cultures were grown in order to check both 10 days immature and 20 days mature virions. All tissues having mutant viral genomes expressed L1 protein, which was similar to tissues containing wild-type viral genomes (Figure 4.7-4.8)

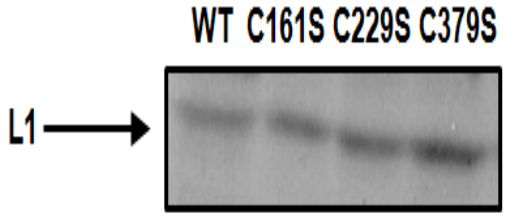

Figure 4.7. No effect of cysteine mutants on HPV16 L1 translation. Western blot analysis of major capsid protein, L1. Showing wild type and other mutant preps. Same volumes of tissue extract were loaded for wild type and mutants.

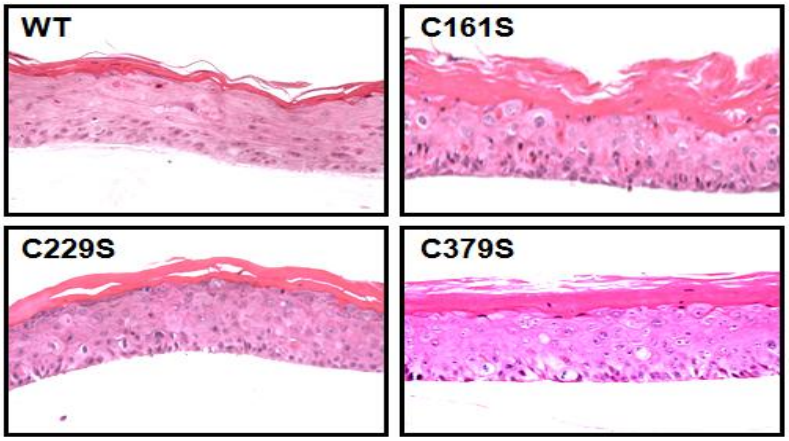

Figure.4.8. No effect of cysteine mutants on HPV-16 growth in organotypic raft tissue culture. Hematoxylin and eosin (H&E) stained wild–type and mutant raft tissues.

The similar levels of genome increase were detected for both wild-type and mutant virus infected cells which shows that cysteine mutants did not affected the on viral genome replication during growth of tissue (Table 4.3).

Table 4.3. HPV16 Viral genome Amplification of wild-type and mutants in differentiating tissue

Virus Type	Fold Change in Amplification
Wild-type	9.60
C161S	10.20
C229S	9.21
C379S	9.83

DNA encapsidation efficiency of wild-type and mutant virions:

Substitution of C161S, C229S, or C379S in HPV16 virus-like particles (VLPs) and pseudo virion (PsV) significantly change their stabilities during purification. In order to quantitatively assess capsid stability, the efficiency of L1 capsid proteins to protect viral genomes in stratified and differentiated epithelial tissues was compared between the wild type and cysteine mutants. This was performed by growing rafts for 10 days and 20 days and then processing into crude viral preps (CVPs). Benzonase treatment was done with CVPs with an aim to remove uncovered viral genomes in the lysate leaving only endonuclease-resistant or encapsidated genomes, or untreated so that total viral genomes in the CVPs could be quantified. With the help of

benzonase treatment, CVPs removed chromatin associated as well as chromatin non associated DNA, through this process small population of resistant viral genome is left for further analysis. Further q-PCR based titration showed that 10-day and 20-day mutants viral titers were 50% or less than 10-day and 20-day titer of wild-type (Fig.4.9). There was not a significant change between titers of 10-day and 20-day wild-type, C161S, C229S, and C379S viral preparations (Fig.4.9). The presence of endonuclease-resistant genomes in mutant viral preps was not as high as compared to wild type preps, which could suggest that it might be a possibility that the mutations at these cysteines can cause early assembly problems, during the first 10 days, for the virus. These data suggest that C161S, C229S, and C379S are critical for proper formation of endonuclease resistant capsids.

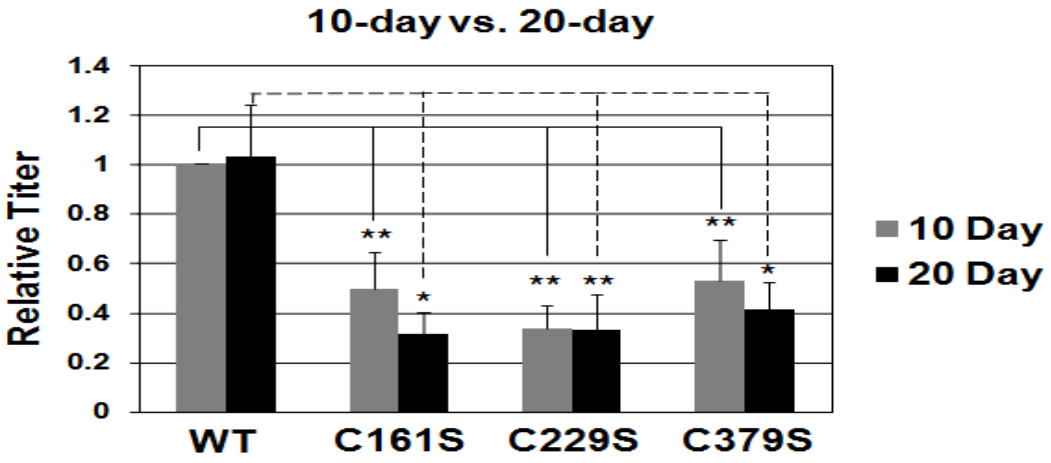

Figure 4.9: Endonuclease-resistant genomes in 10-day and 20-day old HPV16 wild-type and Mutant Crude Viral Preps SYBR green-based DNA encapsidation assay for quantification of 10 and 20-day wild-type (WT), C161S, C229S and C379S CVPs. The WT level was set to 1.0. Benzonase treated samples were quantified by amplifying a viral E2 region using qPCR. The results are means and standard errors for at least three independent experiments.

Relative infectivity of HPV16 wild-type and mutant virions (10- and 20 day)

Considerable decrease in relative endonuclease-resistant genomes were noticed in 10 and 20-day mutant CVPs, all CVPs contained quantifiable endonuclease-resistant genomes. The existence of these genomes suggested that all three mutant L1 proteins helped to protect viral genomes from endonucleases. In order to determine if mutant virions taken from CVPs were infectious or not, infectivity assay based on RT-qPCR was performed. Multiplicity of infection 5 was used for Infections. C161S and C379S mutant virions proved less infectious on 10 and 20 days as compared to wild-type virus (Fig.4.10). The C229S mutant was non-infectious. It was shown earlier by Meyers lab that organotypic culture-derived HPV16 virions utilize a natural host environment's redox gradient that spans the human epithelium. The redox areas of the tissue help virion assembly and maturation in the perspective of the complete papillomavirus life cycle. Comparison of 10- and 20-day viral preps shows that 20-day wild-type virions are more infectious as compared to 10-day wild-type virions (Fig.4.10). Results also showed that C161S and C379S mutants of 20-day were no more infectious than 10-day mutant virus (Fig. 4.10). This shows that mutations to either C161 or C379 stops virions from maturing. C229S is already non-infectious at 10 days culture, which propose that C229 provides a vital step in assembly of virion without which virion remain in a pre-mature state.

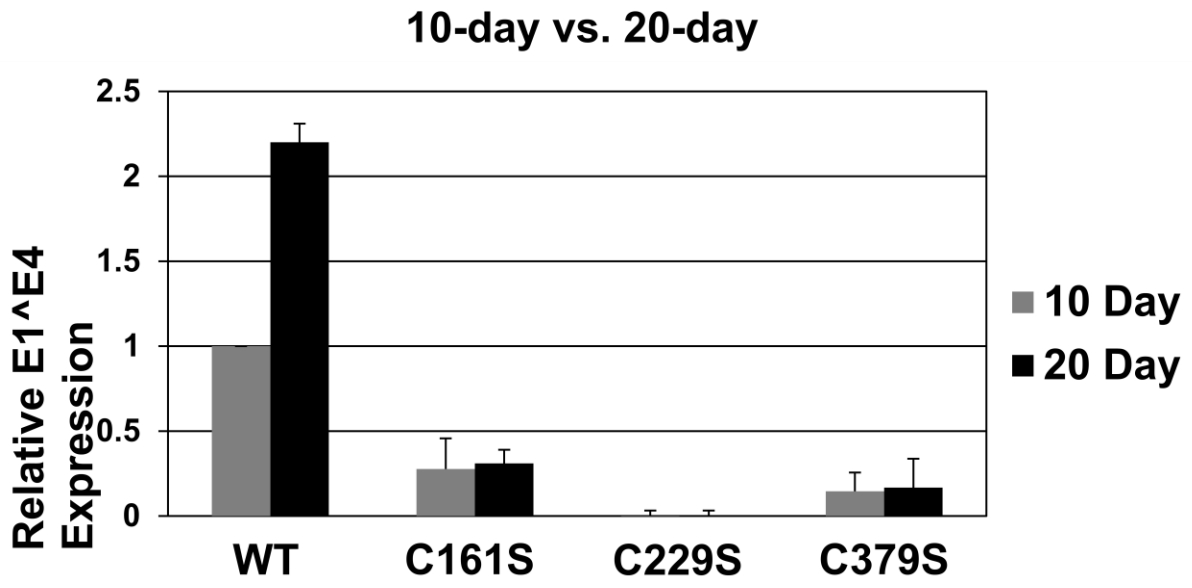

Figure. 4.10. Relative infectivity of HPV 16 wild-type and mutant virions (10 and 20-day). RT-qPCR were used to measure expression level of the E1^E4 spliced transcript by using HaCaT cells after 48 hours post-infection with MOI 5 for both 10- and 20-day tissues.

HPV16 Wild-type and mutant virions capsid stabilities

Optiprep gradient ultracentrifugation was used to determine the capsid stabilities of C161S, C229S, and C379S mutant virus. This Optiprep gradient ultracentifugation helped to make viral genome more prone to endonuclease digestion if capsid is unstable. Ultracentrifugation produces, 11 fractions (1=top and 11=bottom). qPCR were used to measure total genome copies within each fraction. Fractions 1-4 contained separated genomes. These genomes are separated from their capsid as free genomes or are related with disrupted capsids, as re-treatment of these fractions by an endonuclease digests these genomes. Fractions from 6-9 contain those genomes which are protected by their capsids and are resistant to endonuclease treatment. HPV16 wild-type has a genome copy fractionation with 40% of its genomes detected in the most stable fractions, 6-9 (Fig. 4.11).

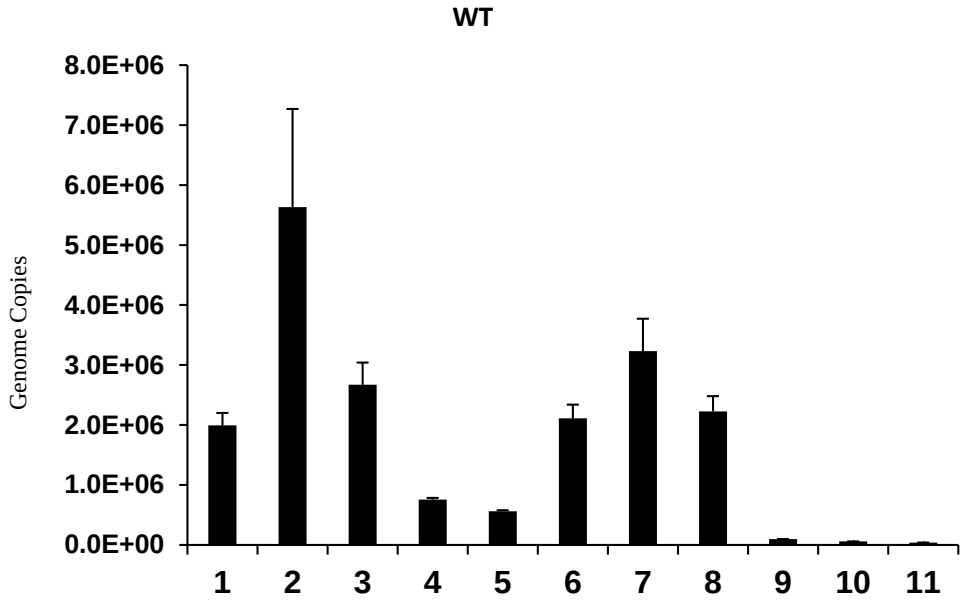

Figure. 4.11 Effect of wild type HPV16L1 on Capsid stability By using ultracentrifuge total genome copies of 20 days viral preps were measured through fractionation. To measure the total genome copies per fraction equal volumes of each fraction were used. With the help of q-PCR Genome copies were quantified by amplifying a region of the viral E2 gene.

The cysteine mutants (C161S, C229S, and C379S) have different genome fractionation profiles, the stable fractions of mutants were fraction number 6-9 with 24.3%, 10.3%, and 18.2% stability respectively (Fig. 4.12A-C)

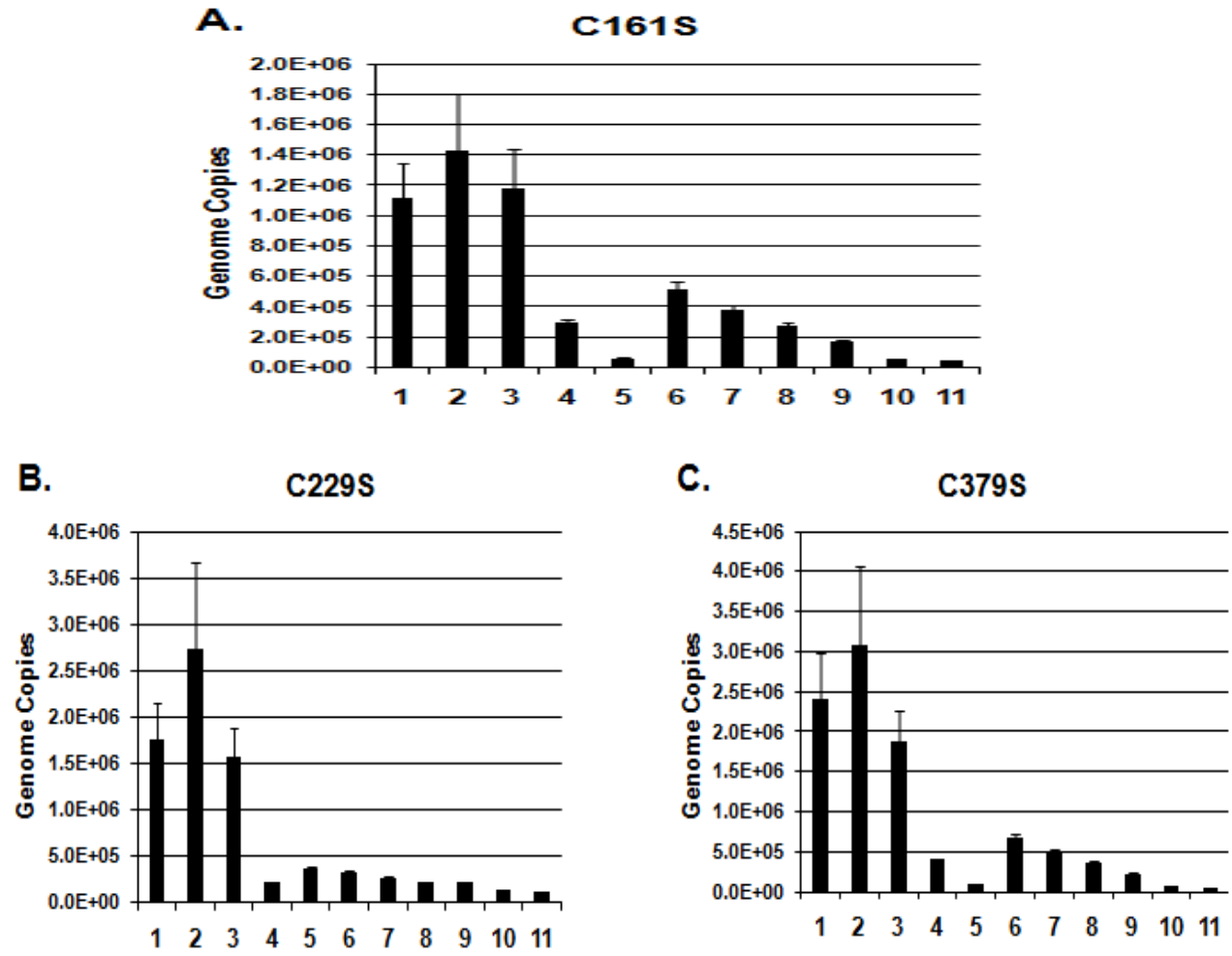

Figure. 4.12 Capsid instability caused by mutation of HPV16 L1 C161, C229, and C379

(A-C) By using ultracentrifuge total genome copies of 20 days viral preps were measured through fractionation. To measure the total genome copies per fraction equal volumes of each fraction were used. With the help of qPCR Genome copies were quantified by amplifying a region of the viral E2 gene.

The relative stability of HPV16 wild-type is approximately 1. Relative stabilities of the cysteine mutants were lower as shown in Table 4.4. These results show that cysteine mutants C161, C229, and C379 play a major role during capsid assembly, which finally give to an increase in capsid stability.

Table 4.4. Relative stability values shown by HPV16 Wild-type and mutant virus

Virus Type	Relative Stability
Wild-type	1.3
C161S	3.0
C229S	7.4
C379S	4.4

Characteristics of fractionated particles

Analyzing the infectivity pattern of each fraction helps to characterize the fractionated particles. Mature wild-type HPV16 virions infectivity was observed in stable fractions 6-9 (Figure 4.13). The three cysteine mutants had lower infectivity than wild-type, with the C379S mutant being the most infectious (Figure 4.13) and this infectivity is generated from unprotected or free genomes.

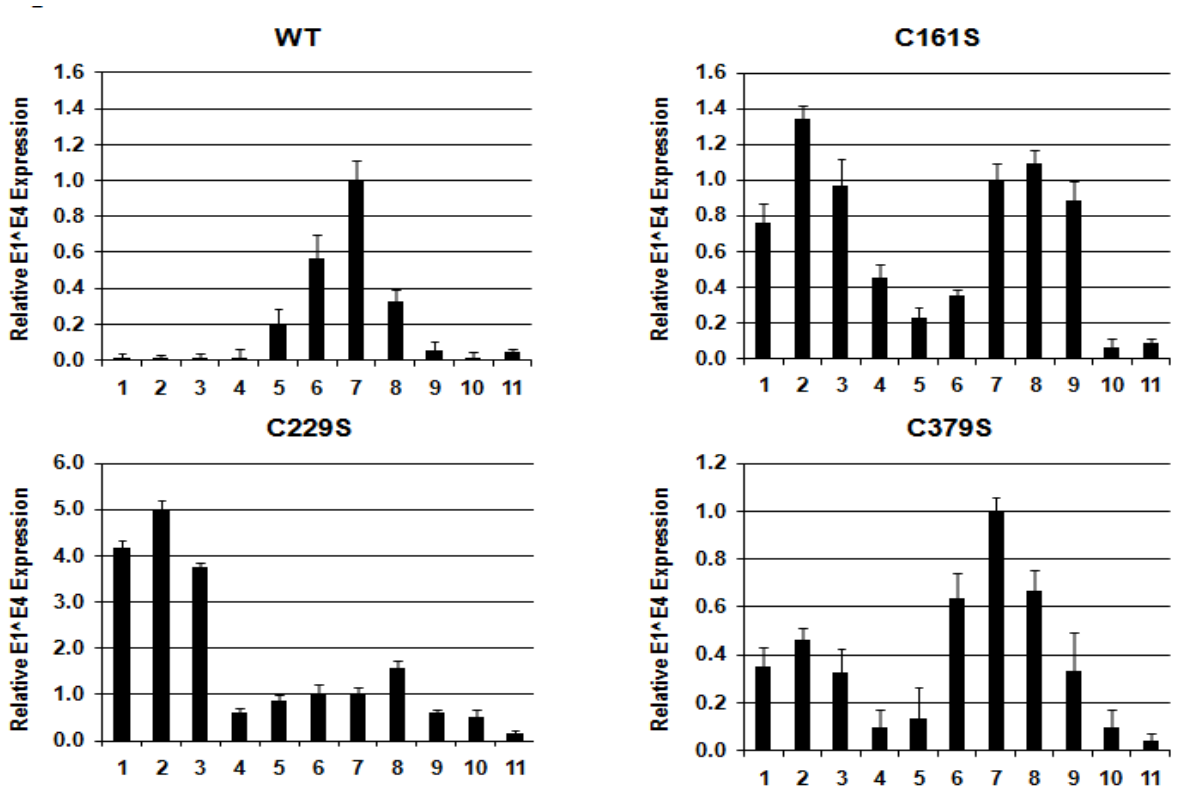

Figure 4.13 Effect of HPV16L1 C161S, C229S, and C379S Mutants on the infectivity profile of fractionated virus. By using ultracentrifuge the relative infectivity (wild-type fraction #7=1) was measured after fractionating viral preps. Equal volumes of each fraction were used to measure the relative infectivity per fraction. It was done by detecting the E1^E4 spliced transcript using RT-qPCR in HaCat cells 48 hours post-infection.

Fraction 2 and fraction 7 samples of wild type and mutants virions were used to compare infectivity at an equal MOI of 5. Less infectivity was shown by all mutants within the stable fractions as compared to wild-type virus at an equal MOI. This results shows that the mutated viral capsids that migrate to the stable fractions are very ineffective to infect their target cells.

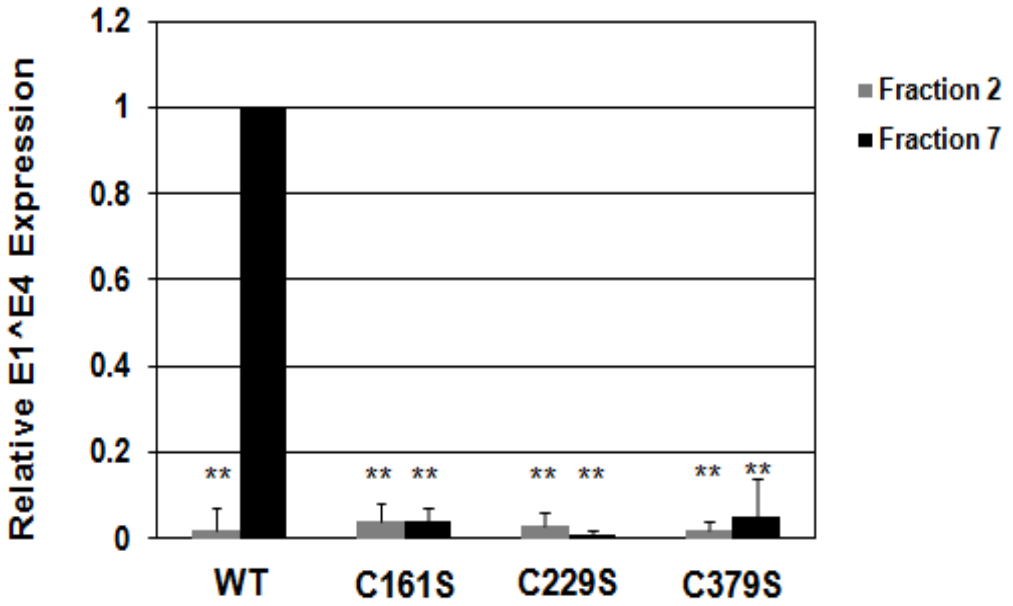

Figure 4.14 Reduced infectivity of virus from stable fractions by HPV16 L1 mutants; C161S, C229S, and C379S Relative levels (wild-type fraction #7=1) of the E1^E4 spliced transcript were measured by RT-qPCR in HaCaT cells 48 hours post-infection at an equal MOI (MOI=5) for fraction #2 and fraction #7 harvested from 20-day tissues.

4.3: Function of upstream Initiation Methionine in the biology of HPV16 L1

Establishment of stable cell lines of WT HPV16 L1, HPV16wtL2-L1, HPV16 1st Met-Ala and HPV16 2ND Met-Ala

For the development of respective producer cell lines primary human foreskin keratinocytes (HFKs) were electroporated with wild-type and site-directed mutagenized HPV16 initiation methionine mutants which were already linearized. Southern blots were performed to confirm the maintenance and recircularization of episomal nature of viral genomes in the producer cell lines of HPV16L1 and 1st and 2nd Met mutants (Figure 4.15).

Mutations inserted in L1 were checked by DNA sequencing (data not shown). After conforming the cell lines have episomal viral DNA they were grown in organotypic raft culture. Organotypic cultures were grown for 10 or 20 days in order to test both immature (10 days) and mature virions (20 days).

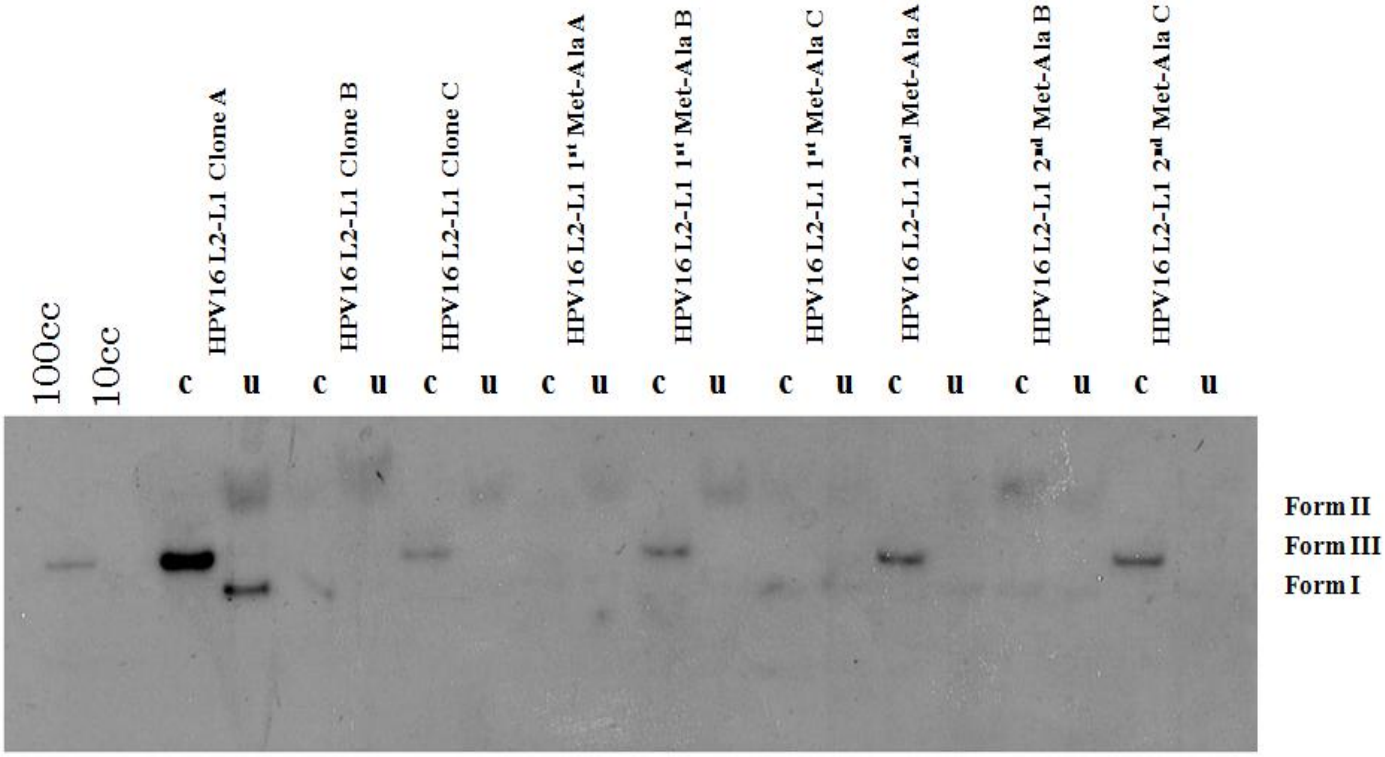

Figure 4.15: Episomal maintenance of wild-type and mutant viral genomes. Southern blot hybridization of HPV16 mutants. Both (U) Uncut and BamHI (C) cut samples shown. The numbers,10 and 100 represent the number of viral genome copies per 5µg of total cellular DNA that was Loaded in lanes u and c. Form 1, supercoiled viral DNA; Form 2,Nicked viral DNA; Form 3,linear viral DNA.

No change in organotypic raft growth because of mutation. Hematoxylin and eosin (H&E) stained Wild-type and mutant raft tissues showed normal growth (Figure 4.16)

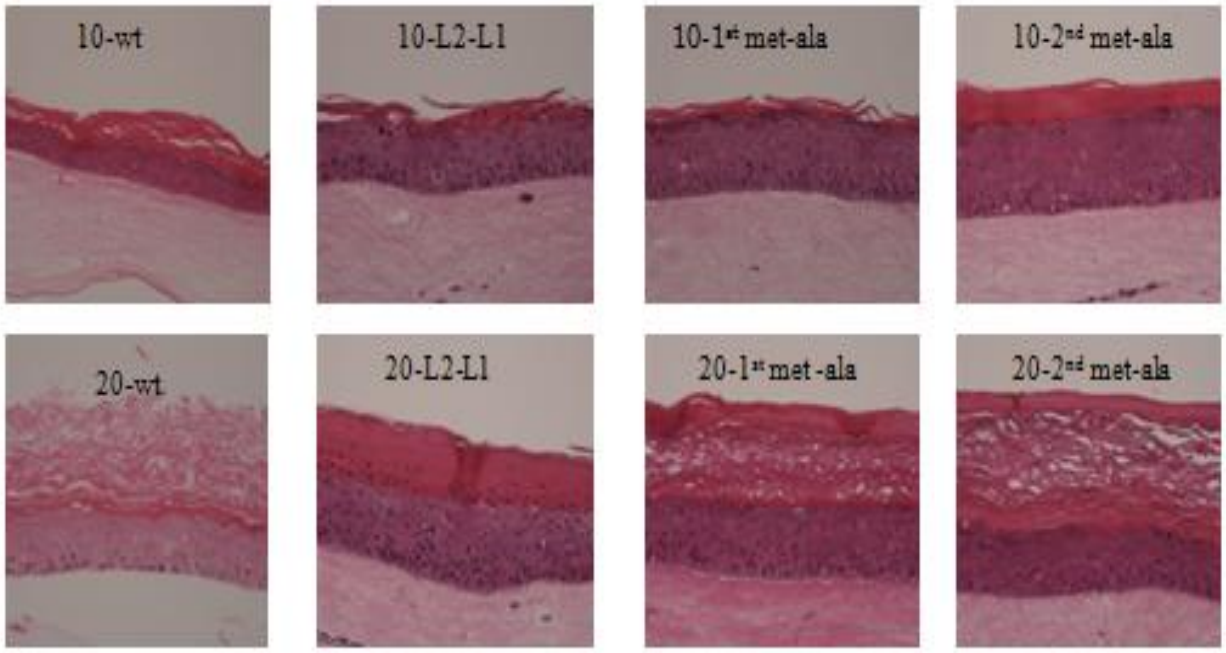

Figure 4.16: Stratification of HPV16wild-type and Met mutants in organotypic cultures.

Hematoxylin and eosin stained paraffin-embedded, 10-day and 20 day wild-type (WT), HPV16L1-L2, HPV16 1st Met-Ala and 2nd Met-Ala infected organotypic tissue.

Table 4.5-4.6 shows Endonuclease-resistant genomes in 10 and 20-day wild-type and mutants CVPs. SYBR green-based DNA encapsidation assay for quantification of 10-day **and 20 day** wild-type (WT), and methionine mutants shown in table 4.5-4.6.

The results are means and standard errors for at least three independent experiments.

Table4. 5 Viral titers from 10-day HPV16 wild-type and Met mutant tissues

Type	**Titer**
HPV16wt	$3.37\ X10^{5}$
HPV16wtL2-L1	$3.97X10^{6}$
HPV16 1st Met-Ala	$1.61X10^{7}$
HPV16 2^{nd} Met-Ala	$3.56X10^{4}$

Table 4.6 Viral titers from 20-day HPV16 wild-type and Met mutant tissues

Type	**Titer**
HPV16wt	$3.37 X10^{5}$
HPV16wtL2-L1	$3.97X10^{6}$
HPV16 1st Met-Ala	$1.61X10^{7}$
HPV16 2^{nd} Met-Ala	$3.56X10^{4}$

Relative infectivity of 20-day wild-type and Met mutant virions

Relative RT-qPCR infectivity assay of 20-day wild-type (WT) and Met Mutants showed that 1^{st} metionine high infectivity and 2^{nd} methionine has low infectivity.

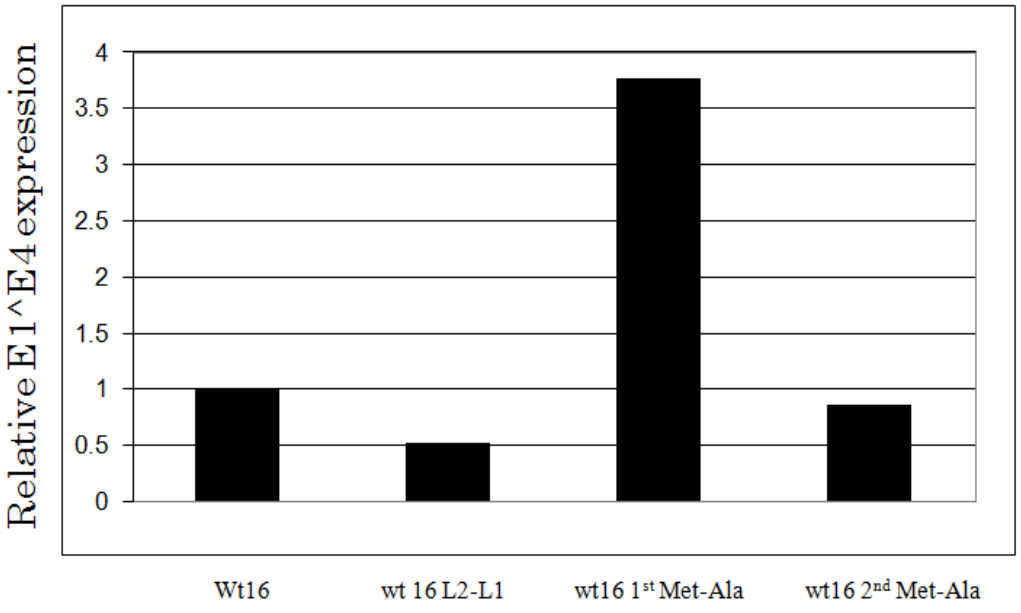

Figure 4.17 Relative infectivity of 20-day wild-type and Met mutant virions.

Relative RT-qPCR infectivity assay of 20-day wild-type (WT) and Met Mutants,

CVPs. The WT value was set to 1.0.

Western blot Analysis of infectious Optiprep fractions:

10 day rafts fractions 7, 8 and 9 were analyzed through western blot.

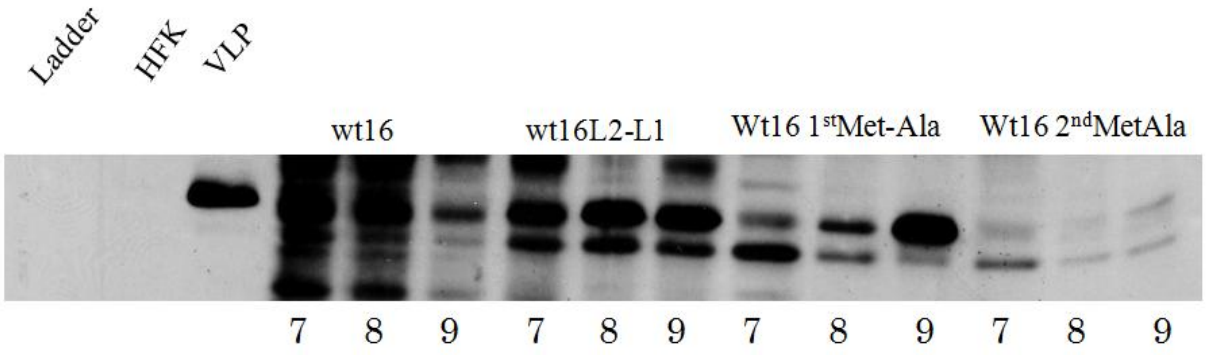

Figure 4.18 Western blot analysis of L1in highly infectious Optiprep fractions no 7, no 8 and no 9 from Optiprep-fractionated 10-day wild-type (WT) and Met mutants.

Western blot Analysis of infectious Optiprep fractions:

20 day rafts fractions 7, 8 and 9 were analyzed through western blot.

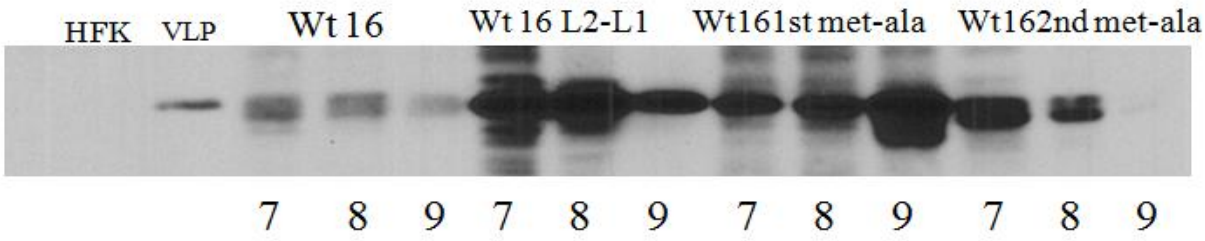

Figure 4.19 Western blot analysis of L1in highly infectious Optiprep fractions no7, no 8 and no 9 from Optiprep-fractionated 20-day wild-type (WT) and Met mutants

Phosphatase treatment of wt16 to analyse the post-translational modification.

It was suggested that these 2 bands of HPV16 native virus (western results) is because of posttranslational modification (phosphorylation) but it was clear from shrimp alkaline phosphatase treatment that these bands are not because of phosphorylation.

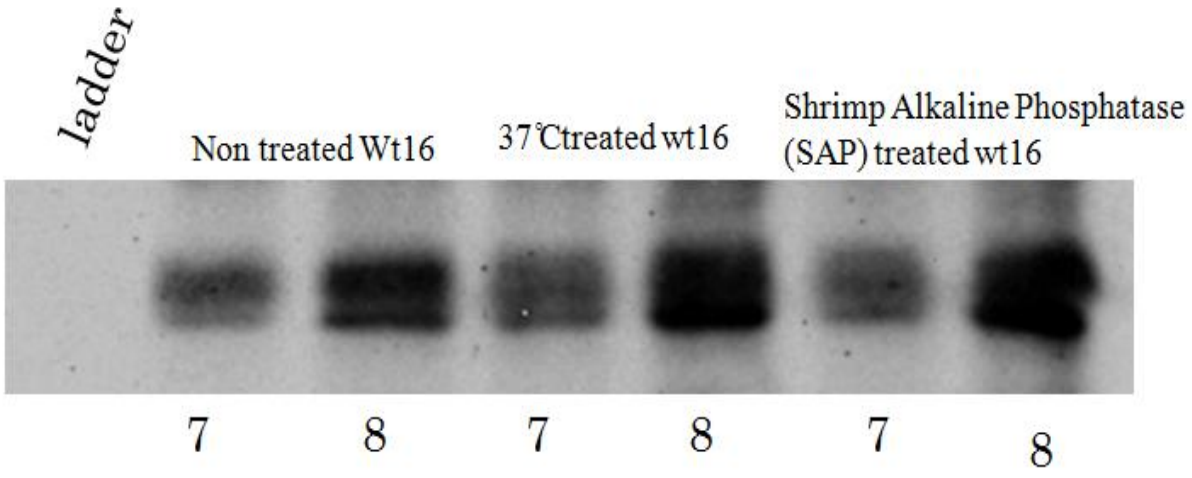

Figure 4.20 shrimp alkaline phosphatase treatment of WT 16 to analyze the role of phosphatases on double bands of HPVwt 16

Discussion

Cervical cancer is the fourth most common gynecological cancer among women worldwide (GLOBOCAN 2012). A wide range of regional diversity in the prevalence of cervical cancer in different parts of the world is clear from several studies. Population-based cancer registries are the only source for calculating the cancer burden in a defined population. Unfortunately, in most developing countries, these programs are either non-existent or are partially functional, consequently preventing the evaluation of actual disease burden (GLOBOCAN 2012). In case of gynecological cancer such as cervical cancer, insufficient health care facilities, deficient cancer registries, lack of routine gynecological screening and HPV testing, and some other factors such as socio-economic issues, cultural believe may deter the actual estimation of the cervical cancer burden (Aniebue *et al* 2014). The conserved societies and beliefs of societies may protect the spread of sexually transmitted disease that is the reason that there is no or very little data available to estimate the HPV-associated disease burden among Pakistani women (Bhurgri *et al* 2004). Pakistan has a growing burden of cervical cancer with changing trends of risk factors associated with it. Approximately 99% of cervical cancer cases are because of high risk HPV infection and approximately 5% of all cancers worldwide (Parkin 2002). High risk HPV is usually a necessary factor for the development of cervical cancer and found in almost every pre-malignant genital lesion (Bosch *et al* 2002; Lombard *et al* 1988). In order to detect HPV in pre-malignant and malignant lesions, PCR based detection methods are in use. Approximately 88 % of all cervical cancer samples and in all cervical intra-epithelial lesions (CIN I-III), HPV DNA was detected. For detection of high risk HPV types, Genotype specific primers HPV 16 (TS 16) and HPV18 (TS 18) were used. HPV 16 was detected in 14/33, 9/18 and 7/8 of Squamous cell carcinoma (SCC), Adenocarcinoma and CIN respectively was detected in 15/33, 7/18 of SCC

and Adenocarcinoma samples. According to the International Association for Research in Cancer (IARC), the most prevalent high risk HPV genotypes found to be related with cervical cancer are: HPV16 (53%), HPV18 (15%), HPV45 (9%), HPV31 (6%), and HPV33 (3%). HPV 16 is the most common subtype throughout the world and usually it is followed by HPV 18 as in many South East Asian countries (Munoz, 2000). The current study shows the occurrence of HPV 16 and 18 in cervical lesions at a comparable rate. HPV 16 and 18 was detected in 42% and 45% of SCC and in 50% and 38.8% of Adenocarcinoma sections respectively (Table 4.1). Seven cervical cancer samples were positive for HPV DNA but not for HPV16 and HPV 18, therefore , they might be infected with HPV oncogenic subtypes other than HPV16 and HPV18(figure 4.4). No HPV DNA was detected in eight cervical cancer samples through GP5/GP6 primer pair. Other studies carried out locally, likewise, reported a minor subpopulation with undetermined genotype (HPV16-/HPV18-). Studies reporting the prevalence of high risk HPV subtypes other than HPV 16 and 18 are inadequate in Pakistan. One of a study carried out in Karachi reported presence of HPV45, HPV56, 59 and 33 in Invasive cervical carcinoma (4.4%, 2.2%, 1.1% and 1.1%) evident of oncogenic HPV subtypes other than HPV 16 and 18 (Raza *et al* 2010). Neighboring countries such as Saudi Arabia reported Major high risk HPV subtypes: HPV 11, 33, 31, 39, 45, 51, 52, 53, 58, 59, 66, 68 and 73 (Alobaid *et al* 2014). In Iran reported data shows HPV subtypes; HPV 6, 11, 31 , 33, 35 ,39, 42, 43, 44, 45 ,51, 52, 56, 58, 59, 66 and 68 (Yousefzadeh *et al* 2014). Most of these studies are based on normal or abnormal cervical screening of the women visiting the gynecologic institutes (Alobaid *et al* 2014; Yousefzadeh *et al* 2014).

Regional differences are present in Pakistan and are evident by different studies. One of a study in Karachi reported HPV prevalence in 18% of cervical cancer samples (Yousaf *et al* 2010).

This study shows the need of a systematic and large scale study. In cervical cancer age plays an important role. Our data establish that more than 50% of cervical cancer cases were in the women aged between 41-60 years of age (Table 4.2/ Figure 4.6). A study carried out in 2010 reported overall low prevalence of HPV infection in general population with no evidence of higher HPV prevalence in young women. Multiple factors are associated with HPV positivity such as women married to either an older man with history of partners, or were in multiple relations (Raza *et al* 2010). The use of prophylactic vaccine can reduce the disease burden. Lack of knowledge about cervical cancer and high costs of vaccine are biggest hurdles in order to lower down the burden of cervical cancer. There is an urgent need of health care programs to educate local people and the Health Care professionals. On the basis of the results, it can be concluded that there is a need for awareness programs, routine gynecological screening and HPV testing in order to determine the HPV related disease burden and its management. Spreading of Knowledge pertaining to the HPV related diseases, risk factors and its prevention to all women is necessary to reduce the disease burden in future.

By knowing the prevalence of HPV and genotyping of High risk HPVs in local population we can promote the use of prophylactic vaccine to reduce HPV related cancers among women of Pakistan. HPV Prophylactic vaccine is based on viral capsid. L1 and L2 genes encode two major proteins of the viral capsid. L1 gene contains many conserved Cysteines and upstream methionines. In this study our next aim was to find out the biological role of Cysteines and Methionines residues in HPV16 biology.

In HPV16L1, Cysteine mutant study, we aimed to determine the roles of highly conserved HPV16 L1 cysteine residues C161, C229, and C379 in the capsid assembly in the environment of stratifying and differentiating human epithelial tissue. For HPV, epithelial tissue

is the natural host environment. Capsids of HPV formed with the help of disulfide bonds formed within the particles as they move through redox gradient present in epithelial tissue of human.

Studies done previously on HPV16 L1 using recombinant particles in monolayer culture and in differentiating tissue system (natural host system) using native virus have clearly shown reliance on inter-pentameric disulfide bonding at C176, C185 and C428 (Ishii *et al* 2003; Conway *et al* 2011). The current study shows the role of cysteine residues C161, C229 and C379 in HPV16 virion assembly in differentiating tissue.

Previous studies comprising of experiments done with VLPs have shown that capsids of cysteine to serine mutants appear structurally related to wild-type capsids when examined under Electron-microscope (EM) (Ishii *et al* 2003). In our studies we observed that in differentiating tissue the cysteine mutants, C161, C229, and C379 produced very low viral titers in 10 and 20 days of tissue growth. This result was surprising, as it was stated previously that these same mutations form VLPs when viewed under EM (Ishii *et al* 2003). Results show that it might be possible that these cysteine play different role within natural environment or that genome encapsidation utilizes these cysteines differently from VLP which have no genome.

Results show that C161S and C379S virions are less infectious. Both mutants show more than 50% decrease in infectivity than the wild type virus in both 10 and 20 days tissue culture. Mutant C229S were non-infectious in both 10 and 20 days tissue culture. Previously it was reported that C229 played a vital role in intracellular trafficking in the period of viral entry, our result support previous studies (Ishii *et al* 2007).

HPV16 wild type virions undergo maturation by utilizing redox tissue gradient of host tissue. They move up in the epithelium for more oxidized environment (Conway *et al* 2009). Same behavior has been shown by recombinant particles which have shown structural changes when

grown in an oxidizing environment (Buck *et al* 2005).This redox environment also play a role in controlling the rate of particle assembly (Mukherjee *et al* 2008).

HPV16 wild-type virions undergo maturation within host tissue, utilizing a redox gradient as they pass up through the epithelium to a more oxidized environment (Conway *et al* 2009). Recombinant particles have also been shown to undergo structural changes when incubated in an oxidizing environment (Buck *et al* 2005). Redox also controls the rate of particle assembly (Mukherjee *et al* 2008). In many viruses such as adenovirus and Human immunodeficiency virus (HIV), maturation has been linked with an increase in its infectivity. Like these viruses HPV16 maturation is also linked with increase in infectivity and this maturation takes place between 10-20 days in organotypic raft culture (Conway *et al* 2009; Joshi *et al* 2006; Perez-berna *et al* 2012). No increase in infectivity was detected when comparing the infectivity level of cysteine mutants, C161S and C379S from 10 and 20 days organotypic raft tissue,(figure 4.10) this suggest that these mutation help in prohibiting maturation of capsid.

Relative stabilities of Cysteine mutants C161S, C229S and C379S are 3.0, 7.4 and 4.4 respectively they all are fragile than wild type virions which have relative stability of 1.3(Table 4.4).

The least affected mutant is C161S and it is 3 time less stable than wild type virion (Table 4.4). It shows that they have the ability to survive in environmental stresses before making a viral progeny. Although these mutants have less stable capsid as compared to wildtype but they have greater relative stabilities than those mutants having inter-pentameric disulfide bonding (Conway *et al* 2011).

Cryo-EM shows that these mutants (C161, C229, and C379) are not involved in the final disulphide bonding which is important for the integrity of mature virion. They are being involved

in inter- pentameric disulfide bonding, we propose that these cysteines can create temporary disulphide bonds during early virus morphogenesis.

There are cysteines in major capsid protein (VP1) of Simian vacuolating virus 40 (SV40) , these cysteines are responsible for inter-pentameric disulfide bonds and also those bonding that affect the final confirmation of capsid in early or late stages during assembly (LiPP *et al* 2005;Gharakhanian *et al* 2005; LiPP *et al* 2002; Jao *et al* 1999; LiPP *et al* 2000). Identification of those cysteines which are involved in brief disulfide bonding is important in order to understand the mechanism of papillomavirus assembly. This data highlights a collective evolutionary approach between papillomaviruses and polyomaviruses to generate a stable capsid that have ability to survive environmental stress.

Finally it is concluded that these data are consistent with an assembly process of HPV 16 that is taking place within differentiated tissue. HPV16L1 C229 was found to be vital for the genome encapsidation as this mutant was found to have the most damaging effect on viral encapsidation, infectivity and stability (Figure 4.10, 4.12-B). It is clear that C229 is utilized a little bit earlier to the virion reaching a pre-mature state early in the assembly process (before 10 days). After 10 days, virion matures. The virion use C161 and C379 residues and if these sites are mutated, it blocks the maturation step. It might be possible that substituting cysteine for serine stop early step of assembly. Relative stability number of these mutations of C161, C229 and C379 shows that they don't impact the structural integrity of the virus as much as other mutants of inter-pentameric disulfide bonding. These cysteines are conserved in many HPV types as they help in the vital processes of coordination of assembly and maturation process of the virus particles within differentiated tissue.

In order to explore the role of two methionine initiation sites in HPV16L1 in the viral biology within the environment of stratifying and differentiating Human epithelial tissue, two mutant organotypic cultures were established. Current research utilizing HPV produced from organotypic raft culture has shown the native viral particle to have different structural characteristics than VLPs. This led to the possibility that one factor causing these differences were the excluded methionines in the VLPs. It is clear from our results that the upstream methionine have important role in the biology of HPV16. It was found that within the context of HPV16 genome, encapsidation was unaffected by the methionine mutations. However the consensus methionine was found to cause an increase in the infectivity compared to wild type. In order to find weather the double bands in wild type HPV16 L1 western blot is because of post translational modification we analyzed the western blot double band of HPV16L1 by treating it with shrimp alkaline phosphatase. Result show that these bands are not because of phosphorylation. Further investigation is needed in this regard.

Conclusion

The study within this thesis has addressed three different aspects regarding high risk human papillomavirus. There is inadequate information regarding HPV prevalence and genotyping among Pakistani women, the first part of the study was to find the most prevalent HPV subtype in paraffin embedded cervical samples collected from local patients. Although this study was performed on relatively smaller cohort of women, it still provides baseline information about HPV prevalence in twin cities of Pakistan. The High risk genotypes HPV16 was found to be the most prevalent subtype. The future studies could also involve the variant analysis which will help to draw precise evolutionary relationship.

The second aspect of this study was to explore the role of conserved molecular moieties in the biology of the most prevalent HPV subtype 16 i.e C161, C229, and C379. It was also found that C161S and C379S mutant virions are more than 50% less infectious than wild-type virus in both 10 and 20 days. C229S mutant virions are non-infectious at either time point. These results suggest a role for C229 in intracellular trafficking during viral entry. No increase in infectivity of both C161S and C379S mutants, suggest that these cysteine residues may have inhibited capsid maturation. Similar experiments can be performed on other HPV subtypes as well, to determine if their assembly kinetics is the same as that of HPV16. First upstream initiation methionine was found to contribute to the viral infectivity which needs further investigation. In conclusion, these findings can be extended in order to design novel early blocking therapeutics.

REFERENCES

Ali SF, Ayub S, Manzoor NF, Azim S, Afif M, Akhtar N, Jafery WA, Tahir I, Farid-Ul Hasnian S, Uddin N., (2010). Knowledge and awareness about cervical cancer and its prevention among interns and nursing staff in Tertiary Care Hospitals in Karachi , Pakistan. PLoS One, 5(6): e11059.

AlObaid A, Al-Badawi IA, Al-Kadri H, Gopala K, Kandeil W, Quint W, Al-Aker M DeAntonio R., (2014). Human papillomavirus prevalence and type distribution among women attending routin gynecological examinations in Saudi Arabia. BMC Infect Dis, 14(1):643.

Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy., (1987). Identification of the HPV16 E6 protein from transformed mouse cells and human cervical carcinoma cell Lines. Embo J, 6:989-92.

Aniebue UU and Onyeka TC., (2005). Ethical, Socioeconomic, and Cultural Considerations in Gynecologic Cancer Care in Developing Countries. International Journal of Palliative Care. Article ID 141627.

Asselineau, D., B. A. Bernard, C. Bailly, M. Darmon, and M. Prunieras., (1986). Human epidermis reconstructed by culture: is it "normal"?. J Invest Dermatol. 86:181-6.

Aziz Z, Sana S, Saeed S, Akram M., (2003). Institution based tumor registry from Punjab: five year data based analysis. J Pak Med Assoc. 53:350–353.

Baay MF, Quint WG, Koudstaal J, Hollema H, Duk JM, Burger MP., (1996). Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. J Clin Microbiol. 3: 745-7.

Badar F, Anwar N, Meerza F, Sultan F., (2007). Cervical carcinoma in a Muslim community.Asian Pac J Cancer Prev. 8: 24–26.

Barksdale, S. K., and C. C. Baker., (1993). Differentiation-specific expression from the bovine papillomavirus type 1 P2443 and late promoters. J Virol. 67: 5605-16.

Banerjee, N. S., N. J. Genovese, F. Noya, W. M. Chien, T. R. Broker, and L.T. Chow., (2006). Conditionally activated E7 proteins of high-risk and low-risk human papillomaviruses induce S phase in postmitotic, differentiated human keratinocytes. J Virol. 80:6517-24.

Bastien, N., and A. A. McBride., (2000). Interaction of the papillomavirus E2 protein with mitotic chromosomes. Virology. 270:124-34.

Bedell, M. A., J. B. Hudson, T. R. Golub, M. E. Turyk, M. Hosken, G. D.Wilbanks, and L. A. Laimins., (1991). Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. J Virol. 65:2254- 60.

Belnap, D. M., N. H. Olson, N. M. Cladel, W. W. Newcomb, J. C. Brown, J.W. Kreider, N. D. Christensen, and T. S. Baker., (1996). Conserved features in papillomavirus and polyomavirus capsids. J Mol Biol. 259: 249-63.

Bhurgri Y. (2004). Karachi Cancer Registry Data–implications for the National Cancer Control Program of Pakistan. Asian Pac J Cancer Prev. 5: 77–82.

Bird, G., M. O'Donnell, J. Moroianu, and R. L. Garcea., (2008). A Possible Role for Cellular Karyopherins in Regulating Polyomavirus and Papillomavirus Capsid Assembly. J Virology.82(20): 9848-57.

Bordeaux, J., S. Forte, E. Harding, M. S. Darshan, K. Klucevsek, and J. Moroianu. (2006). The l2 minor capsid protein of low-risk human papillomavirus type 11 interacts with host nuclear import receptors and viral DNA. J Virol. 80:8259-62.

BoschFX, LorinczA, MunozN, MMeijerC J L, ShahKV., (2002).The causal relation between human papillomavirus and cervical cancer. J Clin Pathol. 55: 244-265.

Bossis, I., R. B. Roden, R. Gambhira, R. Yang, M. Tagaya, P. M. Howley, and P. I. Meneses., (2005). Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. J Virol. 79:6723-31.

Bousarghin, L., A. Touze, P. Y. Sizaret, and P. Coursaget. (2003). Human papillomavirus types 16,31, and 58 use different endocytosis pathways to enter cells.J Virol.77:3846-50.

Broekaert, D., P. Van Oostveldt, P. Coucke, J. De Bersaques, E. Gillis, and P. Reyniers., (1986).Nuclear differentiation and ultimate fate during epidermal keratinization. Two-wavelength and cytofluorometric DNA investigations completed by computerized scanning image analysis. Arch Dermatol Res. 279:100-11.

Brown, D. R., D. Kitchin, B. Qadadri, N. Neptune, T. Batteiger, and A.Ermel., (2006). The human papillomavirus type 11 E1--E4 protein is a transglutaminase 3 substrate and induces abnormalities of the cornified cell envelope. Virology. 345: 290-8.

Bryan, J. T., and D. R. Brown. (2000). Association of the human papillomavirus type 11 E1^E4 protein with cornified cell envelopes derived from infected genital epithelium. Virology. 277: 262-9.

Bryan, J. T., and D. R. Brown. (2001). Transmission of human papillomavirus type 11 infection by desquamated cornified cells. Virology. 281:35-42.

Buck CB, Pastrana DV, Lowy DR, Schiller JT., (2004). Efficient intracellular assembly of papillomaviral vectors. J Virol. 78: 751-757.

Buck, C. B., C. D. Thompson, Y. Y. Pang, D. R. Lowy, and J. T. Schiller., (2005). Maturation of papillomavirus capsids. J Virol. 79: 2839-46.

Buck, C. B., N. Cheng, C. D. Thompson, D. R. Lowy, A. C. Steven, J. T.Schiller, and B. L. Trus., (2008). Arrangement of L2 Within the Papillomavirus Capsid. J Virol. 82: 5190-5197.

Burd EM. (2003). Human Papillomavirus and Cervical Cancer. Clinical Microbiology Reviews. 1–17.

Burgeson, R. E., and A. M. Christiano., (1997). The dermal-epidermal junction. Curr Opin Cell Biol. 9: 651-8.

Campos, S. K., and M. A. Ozbun. (2009). Two highly conserved cysteine residues in HPV16 L2 form an intramolecular disulfide bond and are critical for infectivity in human keratinocytes. PLoS ONE. 4: e4463.

Candi, E., R. Schmidt, and G. Melino., (2005). The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol. 6:328-40.

Chen, X. S., R. L. Garcea, I. Goldberg, G. Casini, and S. C. Harrison., (2000). Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol Cell. 5:557-67.

Chromy, L. R., J. M. Pipas, and R. L. Garcea., (2003). Chaperone-mediated in vitro assembly of Polyomavirus capsids. Proc Natl Acad Sci. 100: 10477-82.

Chromy, L. R., A. Oltman, P. A. Estes, and R. L. Garcea., (2006). Chaperonemediated in vitro disassembly of polyoma- and papillomaviruses. J Virol. 80:5086-91.

Chuong, C. M., B. J. Nickoloff, P. M. Elias, L. A. Goldsmith, E. Macher, P. A. Maderson, J. P. Sundberg, H. Tagami, P. M. Plonka, K. Thestrup-Pederson, B. A. Bernard, J. M. Schroder, P. Dotto, C. M. Chang, M. L.Williams, K. R. Feingold, L. E. King, A. M. Kligman, J. L. Rees, and E.Christophers., (2002). What is the 'true' function of skin? Exp Dermatol. 11: 159-87.

Conger, K. L., J. S. Liu, S. R. Kuo, L. T. Chow, and T. S. Wang. (1999). Human papillomavirus DNA replication. Interactions between the viral E1 protein and two subunits of human dna polymerase alpha/primase. J BiolChem. 274: 2696-705.

Conway MJ, Alam S, Christensen ND, Meyers C., (2009). Overlapping and independent structural roles for human papillomavirus type 16 L2 conserved cysteines. Virology. 393: 295-303.

Conway MJ, Alam S, Ryndock EJ, Cruz L, Christensen ND., (2009). Tissue-spanning redox gradient-dependent assembly of native human papillomavirus type 16 virions. J Virol. 83: 10515-10526.

Conway MJ and Meyers C. (2009). Replication and assembly of human papillomaviruses. J Dent Res. 88: 307-317.

Conway MJ, Cruz L, Alam S, Christensen ND, Meyers C., (2011). Cross-neutralization potential of native human papillomavirus N-terminal L2 epitopes. PLoS One. 6: e16405.

Conway MJ, Cruz L, Alam S, Christensen ND, Meyers C., (2011). Differentiation-dependent interpentameric disulfide bond stabilizes native human papillomavirus type 16. PLoS One. 6: e22427.

Cripe, T. P., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid, 3rd, M.Durst, L. Gissmann, Roman, and L. P. Turek., (1987). Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. Embo J. 6: 3745-53.

Culp, T. D., and N. D. Christensen. (2004). Kinetics of in vitro adsorption and entry of papillomavirus virions. Virology.319: 152-61.

Culp, T. D., N. M. Cladel, K. K. Balogh, L. R. Budgeon, A. F. Mejia, and N.D. Christensen.,(2006). Papillomavirus particles assembled in 293TT cells are infectious in vivo. J Virol. 80:11381-4.

Cumming, R. C., N. L. Andon, P. A. Haynes, M. Park, W. H. Fischer, and D. Schubert., (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem.279: 21749-58.

Day, P. M., D. R. Lowy, and J. T. Schiller., (2003). Papillomaviruses infect cells via a clathrin-dependent pathway. Virology. 307: 1-11.

de Villiers, E. M., C. Fauquet, T. R. Broker, H. U. Bernard, and H. zur Hausen., (2004). Classification of papillomaviruses. Virology.324: 17-27.

de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE.,(2010). RetrospectiveInternational Survey and HPV Time Trends Study Group. Human Papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. Lancet Oncol. 11(11): 1048–1056.

Doorbar, J., D. Campbell, R. J. Grand, and P. H. Gallimore., (1986). Identification of the human papillomavirus-1a E4 gene products. Embo J. 5: 355-62.

Doorbar, J., and P. H. Gallimore., (1987). Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. J Virol. 61:2793-9.

Doorbar, J. (2005). The papillomavirus life cycle. J Clin Virol. 32 1:S7-15.

Dyson, N., P. M. Howley, K. Munger, and E. Harlow., (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science. 243:934-7.

Eckert, R. L., J. F. Crish, and N. A. Robinson., (1997). The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. Physiol Rev.77:397-424.

Eckert, R. L., M. B. Yaffe, J. F. Crish, S. Murthy, E. A. Rorke, and J. F.Welter., (1993). Involucrin--structure and role in envelope assembly. J Invest Dermatol. 100: 613-17.

Elizabeth webb, john cox & stirling Edwards., (2004). Cervical Cancer-causing Human Papillomaviruses have an Alternative Initiation Site for the L1 Protein. Virus Genes. 30: 31–35.

Favre, M., G. Orth, O. Croissant, and M. Yaniv., (1975). Human papillomavirus DNA: physical map. Proc Natl Acad Sci U S A. 72: 4810-4.

Favre, M. (1975). Structural polypeptides of rabbit, bovine, and human papillomaviruses. J Virol.15: 1239-47.

Fay, A., W. H. t. Yutzy, R. B. Roden, and J. Moroianu., (2004). The positively charged termini of L2 minor capsid protein required for bovine papillomavirus infection function separately in nuclear import and DNA binding. J Virol. 78: 13447-54.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Inter- net]. 2010. Lyon,

France,International Agency for Research on Cancer, Available from:http://globocan.iarc.fr.[last accessed April 2012].

Fligge, C., F. Schafer, H. C. Selinka, C. Sapp, and M. Sapp., (2001). DNA induced structural changes in the papillomavirus capsid. J Virol. 75: 7727-31.

Fuchs, E. (1990). Epidermal differentiation: the bare essentials. J Cell Biol. 111: 2807-14.

Fuchs, E., and D. W. Cleveland., (1998). A structural scaffolding of intermediate filaments in health and disease. Science. 279:514-9.

Gambhira, R., B. Karanam, S. Jagu, J. N. Roberts, C. B. Buck, I. Bossis, H. Alphs, T. Culp, N.D. Christensen, and R. B. Roden., (2007). A protective and broadly cross-neutralizing epitope of human papillomavirus L2. J Virol.81:13927-31.

Gharakhanian E, Mana W, Norng M., (2005). Cys254 and Cys49/Cys87of simian virus 40 Vp1 are essential in formation of infectious virions. Virus Res. 107:21-25.

Glick, A. B., A. B. Kulkarni, T. Tennenbaum, H. Hennings, K. C. Flanders, M. O'Reilly, M. B. Sporn, S. Karlsson, and S. H. Yuspa., (1993). Loss of

expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc Natl Acad Sci U S A. 90: 6076-80.

Gloss, B., and H. U. Bernard., (1990). The E6/E7 promoter of human papillomavirus type 16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. J Virol. 64: 5577-84.

Gosselet, F. P., T. Magnaldo, R. M. Culerrier, A. Sarasin, and J. C. Ehrhart., (2007). BMP2 and BMP6 control p57 (Kip2) expression and cell growth arrest/terminal differentiation in normal primary human epidermal keratinocytes. Cell Signal. 19: 731-9.

Hagensee, M. E., N. Yaegashi, and D. A. Galloway., (1993). Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. J Virol. 67: 315-22.

Hagensee, M. E., N. H. Olson, T. S. Baker, and D. A. Galloway., (1994). Threedimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. J Virol. 68: 4503-5.

Hauser, P. J., D. Agrawal, J. Hackney, and W. J. Pledger., (1998). STAT3 activation accompanies keratinocyte differentiation. Cell Growth Differ. 9: 847-55.

Heino, P., J. Zhou, and P. F. Lambert., (2000). Interaction of the papillomavirus transcription replication factor, E2, and the viral capsid protein, L2. Virology. 276: 304-14.

Hindmarsh, P. L., and L. A. Laimins., (2007). Mechanisms regulating expression of the HPV 31 L1 and L2 capsid proteins and pseudovirion entry.Virol J. 4: 19-21.

Hirochika, H., T. R. Broker, and L. T. Chow., (1987). Enhancers and transacting E2 transcriptional factors of papillomaviruses. J Virol. 61: 2599-606.

Holmgren, S. C., N. A. Patterson, M. A. Ozbun, and P. F. Lambert., (2005). The minor capsid protein L2 contributes to two steps in the human papillomavirus type 31 life cycle. J Virol. 79: 3938-48.

Hummel, M., J. B. Hudson, and L. A. Laimins., (1992). Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J Virol. 66: 6070-80.

http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx?cancer=cervix

http://www.hpvcentre.net/statistics/reports/PAK.pdf

Ishii Y, Tanaka K, Kanda T., (2003). Mutational analysis of human papillomavirus type 16 major capsid protein L1: the cysteines affecting the intermolecular bonding and structure of L1-capsids. Virology. 308: 128-136.

Ishii, Y., S. Ozaki, K. Tanaka, and T. Kanda., (2005). Human papillomavirus 16 minor capsid protein L2 helps capsomeres assemble independently of intercapsomeric disulfide bonding. Virus Genes. 31:321-8.

Ishii Y, Kondo K, Matsumoto T, Tanaka K, Shinkai-Ouchi F., (2007) Thiol-reactive reagents inhibits intracellular trafficking of human papillomavirus type 16

pseudovirion by binding to cysteine residues of major capsid protein L1. Virol J. 4: 110-117.

Jao CC, Weidman MK, Perez AR, Gharakhanian E., (1999). Cys9, Cys104 and Cys207 of simian virus 40 Vp1 are essential for inter-pentamer disulfide-linkage and stabilization in cell-free lysates. J Gen Virol. 80 (9): 2481-2489.

Jorcano, J. L., M. Rieger, J. K. Franz, D. L. Schiller, R. Moll, and W. W. Franke., (1984). Identification of two types of keratin polypeptides within the acidic cytokeratin subfamily I. J Mol Biol. 179: 257-81.

Joshi A, Nagashima K, Freed EO., (2006). Mutation of dileucine-like motifs in the human immunodeficiency virus type 1 capsid disrupts virus assembly, gag-gag interactions, gag-membrane binding, and virion maturation. J Virol. 80: 7939-7951.

Kalinin, A., L. N. Marekov, and P. M. Steinert., (2001). Assembly of the epidermal cornified cell envelope. J Cell Sci. 114: 3069-70.

Kanaya, T., S. Kyo, and L. A. Laimins., (1997). The 5' region of the human papillomavirus type 31 upstream regulatory region acts as an enhancer which augments viral early expression through the action of YY1. Virology. 237: 159-69.

Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller., (1992). Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci U S A. 89: 12180-4.

Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Durst, L. Gissmann, D.R. Lowy, and J. T. Schiller., (1993). Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J Virol. 67: 6929-36.

Knight, G. L., A. S. Turnell, and S. Roberts., (2006). Role for Wee1 in inhibition of G2-to-M transition through the cooperation of distinct human papillomavirus type 1 E4 proteins. J Virol. 80: 7416-26

Kondo, K., Y. Ishii, H. Ochi, T. Matsumoto, H. Yoshikawa, and T. Kanda. (2007). Neutralization of HPV16, 18, 31, and 58 pseudovirions with antisera induced by immunizing rabbits with synthetic peptides representing segments of the HPV16 minor capsid protein L2 surface region. Virology. 358: 266-72.

Kyo, S., A. Tam, and L. A. Laimins., (1995). Transcriptional activity of human papillomavirus type 31b enhancer is regulated through synergistic interaction of AP1 with two novel cellular factors. Virology. 211: 184-97.

Larsen, P. M., L. Storgaard, and S. J. Fey., (1987). Proteins present in bovine papillomavirus particles. J Virol. 61: 3596-601.

Lee, Y. S., S. H. Yuspa, and A. A. Dlugosz., (1998). Differentiation of cultured human epidermal keratinocytes at high cell densities is mediated by endogenous activation of the protein kinase C signaling pathway. J Invest Dermatol. 111: 762-66.

Lehman, C. W., and M. R. Botchan., (1998). Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. Proc Natl Acad Sci 95:4338-43.

Li PP, Nakanishi A, Tran MA, Salazar AM, Liddington RC., (2000). Role of simian virus 40

Vp1 cysteines in virion infectivity. J Virol. 74: 11388-11393.

Li PP, Nakanishi A, Clark SW, Kasamatsu H., (2002). Formation of transitory intrachain and interchain disulfide bonds accompanies the folding and oligomerization of simian virus 40 Vp1 in the cytoplasm. Proc Natl Acad Sci 99: 1353-1358.

Li PP, Nakanishi A, Fontanes V, Kasamatsu H., (2005). Pairs of Vp1 cysteine residues essential for simian virus 40 infection. J Virol. 79: 3859-3864.

Lombard I,Vincent-Salomon A, Validire P, Zafrani B, de la Rochefordiere A, Clough K., (1998). Human papillomavirus genotype as a major determinant of the course of cervical cancer. J ClinOncol. 16 (8): 2613–2619.

Longworth, M. S., and L. A.Laimins., (2004). Pathogenesis of humanbpapillomaviruses in differentiating epithelia. Microbiol Mol Biol Rev. 68:362-72.

Lopez, R. G., S. Garcia-Silva, S. J. Moore, O. Bereshchenko, A. B. Martinez- Cruz, O. Ermakova, E. Kurz, J. M. Paramio, and C. Nerlov., (2009).C/EBPalpha and beta couple interfollicular keratinocyte proliferation arrest to commitment and terminal differentiation. Nat Cell Biol. 11: 1181-90.

Lyn TE. Cervical cancer underestimated in Asia: expert http://www.reuters.com/article/2007/11/06/uscancercervixidUSHKG26266720071106

Madison, K. C. (2003). Barrier function of the skin: "la raison d'etre" of the epidermis. J Invest Dermatol. 121: 231-41.

Martinez, L. A., Y. Chen, S. M. Fischer, and C. J. Conti. (1999). Coordinated changes in cell cycle machinery occur during keratinocyte terminal differentiation. Oncogene. 18:397-406.

Matsui, M. S., S. L. Chew, and V. A. DeLeo. (1992). Protein kinase C in normal human epidermal keratinocytes during proliferation and calciuminduced differentiation. J Invest Dermatol. 99: 565-71.

McIndoe, W.A., McLean, M.R., Jones, R.W. and Mullins, P.R.., (1984). The invasive potential of carcinoma in situ of the cervix, Obstetrics and Gynaecology. 64(4):51-458.

McLaughlin-Drubin, M. E., S. Wilson, B. Mullikin, J. Suzich, and C. Meyers., (2003). Human papillomavirus type 45 propagation, infection, and neutralization. Virology. 312:1-7.

McLaughlin-Drubin, M. E., N. D. Christensen, and C. Meyers., (2004). Propagation, infection, and neutralization of authentic HPV16 virus. Virology. 322:213-19.

McPhillips, M. G., J. G. Oliveira, J. E. Spindler, R. Mitra, and A. A.McBride., (2006). Brd4 is required for e2-mediated transcriptional activation but not genome partitioning of all papillomaviruses. J Virol. 80: 9530-43.

Meyers, C., M. G. Frattini, J. B. Hudson, and L. A. Laimins. (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science. 257: 971-73.

Meyers, C., T. J. Mayer, and M. A. Ozbun., (1997). Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. J Virol. 71: 7381-86.

Mischke, D. (1998). The complexity of gene families involved in epithelial differentiation. Keratin genes and the epidermal differentiation complex. Subcell Biochem. 31: 71-104.

Modis, Y., B. L. Trus, and S. C. Harrison., (2002). Atomic model of the papillomavirus capsid. Embo J. 21: 4754-62.

Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan., (1990). Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science. 250: 1694-99

Morris, J. D., T. Crook, L. R. Bandara, R. Davies, N. B. LaThangue, and K.H. Vousden ., (1993). Human papillomavirus type 16 E7 regulates E2F and contributes to mitogenic signalling. Oncogene. 8: 893-98.

Mukherjee S, Thorsteinsson MV, Johnston LB, DePhillips PA, Zlotnick A., (2008). Aquantitative description of in vitro assembly of human papillomavirus 16 virus-like particles. J Mol Biol. 381: 229-237.

Mulazim Hussain Bukhari, Muhammad Muddasar Majeed, Samina Naeem, Shahida Niazi, Samina Qamar, Kanwal Saba., (2012). Clinicopathological importance of Papanicolaou Smears for The diagnosis of premalignant and malignant lesions of the cervix. Journal of Cytology. 29(1): 20-25.

Munoz, N. (2000). Human papillomavirus and cancer: the epidemiological evidence. J. Clin.Virol. 19: 1–5.

Neary, K., and D. DiMaio. (1989). Open reading frames E6 and E7 of bovine papillomavirus type 1 are both required for full transformation of mouse C127 cells. J Virol. 63: 259-66.

Ozbun, M. A., and C. Meyers. (1997). Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. J Virol.71: 5161-72.

Ozbun, M. A., and C. Meyers. (1998). Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. Virology. 248:218-30. (a)

Ozbun, M. A., and C. Meyers. (1998). Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. J Virol .72: 2715-22. (b)

Ozbun, M. A., and C. Meyers. (1999). Two novel promoters in the upstream regulatory region of human papillomavirus type 31b are negatively regulated by epithelial differentiation. J Virol. 73:3505-10.

Ozbun, M. A. (2002). Infectious human papillomavirus type 31b: purification and infection of an immortalized human keratinocyte cell line. J Gen Virol. 83: 2753-63.

Pang, Y. Y., A. Schermer, J. Yu, and T. T. Sun., (1993). Suprabasal change and subsequent formation of disulfide-stabilized homo- and hetero-dimers of keratins during esophageal epithelial differentiation. J Cell Sci. 104 (3): 727-40.

Parkin DM. (2006). The global health burden of infection-associated cancers in the year 2002. International Journal of Cancer. 118(12): 3030–3044.

Pastrana, D. V., R. Gambhira, C. B. Buck, Y. Y. Pang, C. D. Thompson, T. D. Culp, N. D. Christensen, D. R. Lowy, J. T. Schiller, and R. B. Roden., (2005). Cross-neutralization of cutaneous and mucosal Papillomavirus types with anti-sera to the amino terminus of L2. Virology. 337: 365-72.

Patterson, N. A., J. L. Smith, and M. A. Ozbun., (2005).Human papillomavirus type 31b infection of human keratinocytes does not require heparan sulfate. J Virol. 79: 6838-47.

Perez-Berna AJ, Ortega-Esteban A, Menendez-Conejero R, Winkler DC, Menendez M., (2012). The role of capsid maturation on adenovirus priming for sequential uncoating. J Biol Chem. 287: 31582-31595.

Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley., (1988). The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell. 53:539-47.

Pillai, S., D. D. Bikle, M. L. Mancianti, and M. Hincenbergs., (1991). Uncoupling of the calcium sensing mechanism and differentiation in squamous carcinoma cell lines. Exp Cell Res. 192: 567-73.

Poumay, Y., and M. Leclercq-Smekens. (1998). *In Vitro* models of epidermal differentiation. Folia Med (Plovdiv). 40:5-12.

Prunieras, M., M. Regnier, and D. Woodley., (1983). Methods for cultivation of keratinocytes with an air-liquid interface. J Invest Dermatol. 81:28s-33s.

Pyeon, D., P. F. Lambert, and P. Ahlquist., (2005). Production of infectious human papillomavirus independently of viral replication and epithelial cell differentiation. Proc Natl Acad Sci. 102: 9311-6.

Querleux, B., C. Cornillon, O. Jolivet, and J. Bittoun., (2002). Anatomy and physiology of subcutaneous adipose tissue by in vivo magnetic resonance imaging and spectroscopy relationships with sex and presence of cellulite. Skin Res Technol. 8: 118-24.

Raza SA, Franceschi S, Pallardy S, Malik FR, Avan BI, ZafarA., (2010). Human papillomaviru infection in women with and without cervical cancer in Karachi, Pakistan. Br. J. Cancer.102, 1657–1660

Rice, R. H., and H. Green. (1978). Relation of protein synthesis and transglutaminase activity to formation of the cross-linked envelope during terminal differentiation of the cultured human epidermal keratinocyte. J Cell Biol. 76:705-11.

Roden, R. B., A. Armstrong, P. Haderer, N. D. Christensen, N. L. Hubbert, D. R. Lowy, J. T. Schiller, and R. Kirnbauer., (1997). Characterization of a human papillomavirus type 16 variant-dependent neutralizing epitope. J Virol. 71:6247-52.

Rolfe, M., P. Beer-Romero, S. Glass, J. Eckstein, I. Berdo, A. Theodoras, M. Pagano, and G. Draetta., (1995). Reconstitution of p53-ubiquitinylation reactions from purified components: the role of human ubiquitinconjugating enzyme UBC4 and E6-associated protein (E6AP). Proc Natl Acad Sci. 92: 3264-8.

Romanczuk, H., F. Thierry, and P. M. Howley.,(1990). Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. J Virol. 64: 2849-59.

Ruesch, M. N., F. Stubenrauch, and L. A. Laimins.,(1998). Activation of papillomavirus late gene transcription and genome amplification upon differentiation in semisolid medium is coincident with expression of involucrin and transglutaminase but not keratin-10. J Virol. 72: 5016-24.

Sapp, M., C. Fligge, I. Petzak, J. R. Harris, and R. E. Streeck., (1998).Papillomavirus assembly requires trimerization of the major capsid protein by disulfides between two highly conserved cysteines. J Virol. 72:6186-9

Selinka, H. C., T. Giroglou, T. Nowak, N. D. Christensen, and M. Sapp., (2003). Further evidence that papillomavirus capsids exist in two distinct conformations. J Virol. 77: 12961-70.

Smith, J. L., S. K. Campos, and M. A. Ozbun., (2007). Human papillomavirus type 31 uses a caveolin 1- and dynamin 2-mediated entry pathway for infection of human keratinocytes. J Virol. 81: 9922-31.

Smith, J. L., S. K. Campos, A. Wandinger-Ness, and M. A. Ozbun., (2008). Caveolin-1 dependent infectious entry of human papillomavirus type 31 in human keratinocytes proceeds to the endosomal pathway for pH-dependent uncoating. J Virol. 85: 9956-62

Swindle, C. S., N. Zou, B. A. Van Tine, G. M. Shaw, J. A. Engler, and L. T. Chow., (1999). Human papillomavirus DNA replication compartments in a transient DNA replication system. J Virol. 73: 1001-9.

Trus, B. L., R. B. Roden, H. L. Greenstone, M. Vrhel, J. T. Schiller, and F. P.Booy., (1997). Novel structural features of bovine papillomavirus capsid revealed by a three-dimensional reconstruction to 9 A resolution. Nat Struct Biol. 4:413-20.

Ura, H., F. Takeda, and H. Okochi., (2004). An in vitro outgrowth culture system for normal human keratinocytes. J Dermatol Sci. 35:19-28.

Volpers, C., P. Schirmacher, R. E. Streeck, and M. Sapp., (1994). Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. Virology. 200: 504-12.

Walboomers J.M.M., Jacobs, M.V., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., Snijder, P.J., Peto, J., Meijer, C.J . and Munoz, Nl., (1999). Human papillomavirus is a necessary cause of invasive cancer worldwide, Journal of pathology. 189: 12–19.

Wang, Q., H. Griffin, S. Southern, D. Jackson, A. Martin, P. McIntosh, C. Davy, P. J. Masterson, P. A. Walker, P. Laskey, M. B. Omary, and J.Doorbar., (2004). Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for *in vivo* and *in vitro* keratin filament reorganization. J Virol. 78:821-33.

Wilson, R., and L. A. Laimins. (2005). Differentiation of HPV-containing cells using organotypic "raft" culture or methylcellulose. Methods Mol Med. 119:157-69.

Wright DK and Manos MM. Sample preparation from paraffinembedded tissues, in Polymerase chain reaction protocols: a guide to methods and applications p. (*Innis,* M. A., D, H.Gelfand, and J. J.Sninsky andwhite ,T.J.,ds.),Academic, Newyork 1990;pp.153-158.

Yi, R., M. N. Poy, M. Stoffel, and E. Fuchs., (2008). A skin microRNA promotes differentiation by repressing 'stemness'. Nature. 452: 225-9.

Yoon, C. S., K. D. Kim, S. N. Park, and S. W. Cheong., (2001). alpha(6)Integrin is the main receptor of human papillomavirus type16 VLP. Biochem Biophys Res Commun. 283:668- 73.

You, J., J. L. Croyle, A. Nishimura, K. Ozato, and P. M. Howley., (2004).Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. Cell. 117:349-60.

Yousefzadeh A, Mostafavizadeh SM, Jarollahi A, Raeisi M, Garshasbi M, Siavashvahabi Z, Moazzami B, Shafaghi B. (2014). Human papillomavirus (HPV) prevalence and types among Women attending regular gynecological visit in Tehran, Iran. Clin Lab. 60(2): 267-73.

Yousuf S, Syed S, Moazzam A, Lucky MH. (2010). Frequency of high risk human papillomavirus types in squamous cell carcinoma of cervix among women. Journal of Pakistan medical association. 60(3):103-196.

Zhao, K. N., K. Hengst, W. J. Liu, Y. H. Liu, X. S. Liu, N. A. McMillan, and I. H. Frazer., (2000). BPV1 E2 protein enhances packaging of full-length plasmid DNA in BPV1 pseudovirions. Virology. 272: 382-93.

Zheng, Z. M., and C. C. Baker., (2006). Papillomavirus genome structure, expression, and post- transcriptional regulation. Front Biosci. 11: 2286-302.