Identification and Characterization of Human Papillomavirus Causing Cervical Cancer in Pakistani Women

By

Amama Rani

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2021

Identification and Characterization of Human Papillomavirus Causing Cervical Cancer in Pakistani Women

A thesis submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

In

Microbiology

By

Amama Rani

Department of Microbiology

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2021

Author's Declaration

I **Ms. Amama Rani** hereby state that my Ph.D. thesis titled **"Identification and Characterization of Human Papillomavirus Causing Cervical Cancer in Pakistani Women"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Ms. Amama Rani
Date: 22-02-2021

Plagiarism Undertaking

"Identification and Characterization of Human Papillomavirus Causing Cervical Cancer in Pakistani Women" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D. degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature:

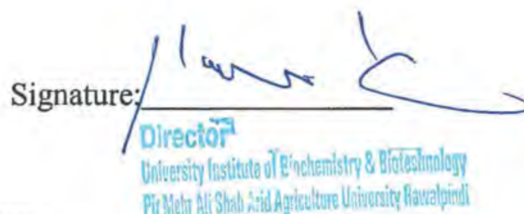
Name: **Ms. Amama Rani**

Certificate of Approval

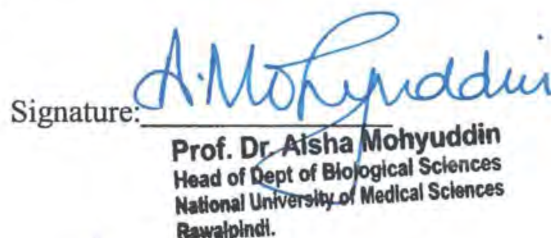
This is to certify that the research work presented in this thesis, entitled titled **"Identification and Characterization of Human Papillomavirus Causing Cervical Cancer in Pakistani Women"** was conducted by **Ms. Amama Rani** under the supervision of **Prof. Dr. Fariha Hasan.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Ms. Amama Rani** Signature:

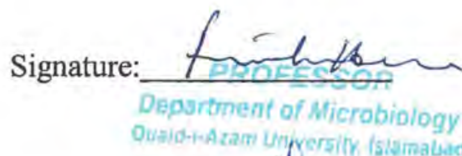
Examination Committee:

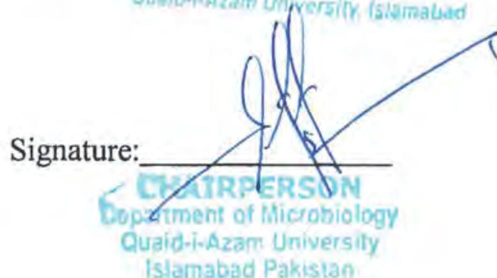
a) External Examiner 1: Signature: 

Dr. Ghazala Kaukab Raja
Professor and Director
University Institute of Biochemistry & Biotechnology,
PMAS Arid Agriculture University, Murree Road,
Rawalpindi

b) External Examiner 2: Signature: 

Prof. Dr. Aisha Mohyuddin
National University of Medical Sciences
The Mall, Abid Majeed Road, Rawalpindi

Supervisor Name: **Prof. Dr. Fariha Hasan** Signature: 

Name of HOD: **Dr. Aamer Ali Shah** Signature: 

Dedicated to

My Loving Mothers

Prof. Fatima Rani (Late)

&

Prof. Dr. Khadija Ali (Shaheed)

To whom I owe the sweet memories,

who are no more with me,

to see the bud of their wishes and

prayers bloom in to flower.

&

My Loving Father

S. A. Latif Khan Chughtai

His sacrifice sympathizes and prayers,

who is my hope in miser,

my consolation in sorrows and

for what he made me.

Acknowledgment

First and foremost I would like to thank "Allah Almighty" whose help in completing this research is one of his great blessings on me. Countless salutations upon the Holy Prophet Hazrat Muhammad (P. B. U. H) who enabled me to recognize my creator and guided mankind on the true path of life.

This thesis has proven to be challenging and thus it is benefiting for me to mention how grateful I am to some of the most grateful people I am lucky to have met, who showed me important virtues in the journey.

Among them first of all I am deeply indebted to Prof. Dr. Fariha Hasan, my supervisor for the invaluable guidance and constructive remarks throughout my research. Though I fear that these lines are very small tribute to all she has done for my academic as well as moral support. I am deeply indebted for her guidance, precious advices and genuine interest, constructive criticism and generous supervision throughout my research work. This tedious task would not be possible without her co-operation, kind interest and moral encouragement.

It is privilege and honor to express my deepest gratitude to all my kind hearted and respected teachers and staff of Department of Microbiology for their co-operation.

This thesis could not have come about without the help of Molecular biology lab, virology Department National Institute of Health Islamabad. My gratitude goes to Dr. Syed Sohail Zahoor Zaidi and Dr. Shahzad Shaukat for their generous support and guidance. I would like to thank all the staff members, former and present in particular, making lab a nice environment were to work.

Deep indebtedness and cordial thanks are also due to Colonel Eijaz Ghani; Department of Virology and Brigadier Shoaib Naiyar Hashmi; Department of Pathology, Armed Forces Institute of Pathology Rawalpindi, Dr. Ahmareen Khalid Sheikh; Histopathology Section, Pathology Department Pakistan Institute Of Medical Sciences, Islamabad and Dr. Nasira Tasneem; Department of Gynecology and Obstetrics Pakistan Institute Of Medical Sciences, Islamabad for extending cooperation and generous help and valuable guidance during sample collection.

I am extremely grateful to Dr. John Doorbar, Professor and Director of Human papillomavirus Research Group, University of Cambridge, United Kingdom

for providing research facilities to carry out my research project in his laboratory and also for his cooperation, useful suggestions, inspiration, and counseling from time to time to complete this research project.

It is my pleasure to mention Dr. Heather, Dr. Nagayasu, Dr. Christian, Dr. Isao, Samantha, Diane, Karen for their great cooperation. I believe that without their help my Ph.D. project may not have been furnished so quickly and nicely. I am also extremely grateful to my Land Lords Dr. Richard Higginson and Felicity Higginson and my housemate Chandani Bahana for their kindness and support during my stay in far land. I would like to express my heartiest thanks to my friends Attiya, Samra and Anham.

Last but certainly not least, the biggest "thank you" goes to my family, Ama, my Amen api, Javed bhai, Jawad bhai, Dr. Jiya Rani, Maj. Saad, Maj. Dr. Rabia. My beloved nephews and nieces Anayah, Arsal, Areeba, Abdullah, Rayyan, Abdul Moeed, Azlan and Abeeha who always pray for me and who did not let me feel alone by sending me their cheerful and encouraging messages. I should like to record thanks and appreciation for their support and unfailing patience and tolerance of my absence.

Above all my loving **"Daddy Jee"** who offered continual prayers, encouragement moral support motivating me during indefinite stage in my life. **This product is dedicated to him in a very special way.**

Finally all errors that remains, are mine, alone.

Amama Rani

Abstract

Cervical cancer is the most prevalent gynecological malignancy in low to middle-income countries. In Pakistan, cervical cancer ranks as the 3rd leading cause of death in females. The important reason for this higher mortality rate in country is due to lack of valuable screening programs. Cancer of the cervix is a preventable disease. In Pakistan, HPV screening is relatively neglected and information on HPV prevalence and its molecular epidemiology is limited among Pakistani women.

The purpose of this study was to determine the socio-demographic factors influencing cervical cancer screening and to assess the role of screening should be test for early detection of cervical carcinoma in the Pakistani female. Beside this, molecular characterization, genotyping and detection of human papillomavirus from formalin fixed paraffin embed cervical biopsies obtained from patients with cervical cancer to determine most prevalent HPV type in Pakistan. Furthermore, it demonstrates additional information regarding role of HPV infection in early events of epithelial wound healing an *in vitro* model system.

Between, January 2013 to December 2014 cervical smears were taken from married females with gynecological problems visiting Pakistan Institute of Medical Sciences. Pap smears were also obtained from Armed Forces Institute of Pathology, Rawalpindi. In the present study, a total of 118 cervical smears were screened using Pap smear test. All patients were also subjected to visual inspection with acetic acid (VIA). The mean age of the female patients was 35.78 ± 9.8 years. There were 42(35.5%) normal Pap smear, 33(27.9%) inflammatory, 11(9.3%) LSIL,5(4.2%)HSIL and 18(15.2%) abnormal Pap smear. Acetic acid (VIA) was positive in 27/118 (22.8%) patients.

The present study also determined and characterized HPV infections and to evaluated the HPV DNA in subjects with abnormal cervical cytology. A total of 105 histopathological confirmed formalin fixed paraffin embedded (FFPE) biopsies were obtained from cervical cancer patients and were screened for HPV DNA by nested PCR using primers MY09/MY11 and GP5+/GP6+. The HPV positive samples were also screened for high risk HPV genotypes by HPV genotyping PCR and further confirmed and characterized by nucleotide sequencing. HPV DNA was

detected in 90 (96.8 %) FFPE biopsies. Out of positive HPV, 81 (90%) and 1 (1.2%) were infected with HPV16 and HPV18 respectively, while 8 (8.8 %) remained untypeable on genotyping HPV PCR. DNA sequencing revealed HPV16 (n= 41) was the most frequent type followed by HPV35, 18, 56, 59, 67 and 73 respectively. To the best of our knowledge, molecular epidemiology of HPV 35, 56, 59, 67 and 73 were first time reported in Pakistan. This study provides important information on the HPV prevalence and its high-risk type distribution causing cervical cancer in Pakistan. It contributes information related to molecular epidemiology of HPV types in the country that will be fundamental for local decision-makers to consider cervical cancer screening programs and useful globally for the understanding of HPV variant distribution around the world

Another important part of present study was establishment of Normal Immortalized Human Keratinocytes (NIKS) and NIKS HPV 16 cell lines to know early events of HPV infection and wound healing process and to determine role of epidermal growth factor (EGF). As EGF is an important proliferative agent and is necessary in tissue culture experiments to stimulate the proliferation of NIKS cells. HPV 16 viral copy number increased when using 500 ng/ml, hence high EGF increase in viral copy number but do not have impact on morphology of keratinocytes and proliferation of cells. Expression levels of E6 indicate >2-3 fold increase at day 5 in the 100ng/ml and 500ng/ml samples respectively.

The findings of this thesis have great implementation and provide an over view on prevalence, molecular epidemiology and *in vitro* model studies. Therefore, it is need of time to monitor virus for early detection and better characterization which will not only help the physicians, surgeons, oncologist for rapid diagnosis but also help researchers for investigating early events in viral infection and wound healing process.

TABLE OF CONTENTS

Acknowledgment i
Abstract iii
TABLE OF CONTENTS v
LIST OF TABLES ix
LIST OF FIGURES x
List of abbreviations xiii
1. INTRODUCTION 1
Aim and objectives 6
2. REVIEW OF LITERATURE 7
2.1 Human papillomavirus and associated diseases 7
2.1.1 Cancer of head and neck 7
2.1.2 Anogenital warts 7
2.1.3 Cancer of penis 7
2.1.4 Cancer of anus 8
2.1.5 Cancer of vulva and vagina 8
2.1.6 Cancer of cervix 8
2.2 Incidence of cervical cancer caused by Human papillomavirus 8
2.2.1 Incidence of cervical cancer in Asia 9
2.2.2 Cervical cancer incidence in Islamic Republic of Pakistan. 10
2.3 History of discovery of human papillomaviruses (HPV) 10
2.4 Classification of papillomaviruses 12
2.5 Structure of human papillomavirus 14
2.6 Genome of Human Papillomavirus 15
2.7 HPV gene expression 16
2.7.1 HPV gene transcription 16
2.7.2 Translation in HPV mRNAs 19
2.8 Risk factors of HPV infection and cervical cancer 19
2.8.1 Sexually transmitted infection 19
2.8.2 Age 20
2.8.3 Early Marriage and pregnancy 20
2.8.4 Parity 20
2.8.5 Multiple sexual partners 20

2.8.6 Impaired immune system 20
2.8.7 Use of oral contraceptives 21
2.8.8 Smoking 21
2.8.9 Socio-economic factors 21
2.9 HPV infection transmission 22
2.9.1 Horizontal transmission 22
2.9.2 Vertical Transmission 22
2.10 Physiology of the uterine cervix and HPV infection .... **Error! Bookmark not defined.**
2.11 Life cycle of Human Papilloma virus 23
2.11.1 Early Events 25
2.11.2 Late Events 27
2.12 Pathology grading of Human papilloma virus lesion 28
2.13 Immune response and clearance of HPV infection 30
2.14 Wound healing process 30
2.14.1 Growth factors involve in wound healing 31
2.14.2 Role of Epidermal growth factor in wound healing 32
2.15 Detection of Human papilloma virus 34
2.15.1 Screening of HPV associated lesion 34
2.15.2 Molecular methods for HPV detection 35
2.16 Prevention 37
2.16.1 Primary prevention 37
2.16.2 Secondary prevention 38
2.17 Vaccines 38
2.18 Treatment 39
3. Materials and Methods 40
3.1 Phase 1: Evaluation of socio-demographic characteristics 40
3.1.1 Study Population 40
3.1.2 Ethical approval 40
3.1.3 Demographic data collection 40
3.1.4 Data analysis 42
3.2 Phase 2: Pelvic examination and Screening test 42
3.2.1 Inclusion and exclusion criteria 42
3.2.2 Pelvic examination and sample collection 42
3.2.3 Reporting of Pap smears 43
3.3 Phase 3: HPV Screening and genotyping 44

3.3.1 Study conducted ... 44
3.3.2 Sample collection ... 44
3.3.3 Extraction of Genomic DNA and Detection of HPV Infection ... 45
3.3.4 Polymerase chain reaction for amplification of β globin gene ... 45
3.4 Phase 4: DNA sequencing ... 48
3.4.1 Polymerase chain reaction for HPV ... 48
3.4.2 Purification of amplified products ... 48
3.4.3 Cycle sequencing/Nucleotide sequencing PCR ... 49
3.4.4 Purification of DNA sequencing samples ... 49
3.4.5 Sequence analysis ... 50
3.5 Phase 5: Keratinocyte and fibroblast monolayer cell culture ... 50
3.5.1 Buffer and reagents ... 51
3.5.2 Basic monolayer cell culture method ... 51
3.5.3 Quantification of genomic DNA ... 56
3.6 Phase 6: Monolayer growth assay for determining effect of epidermal growth factor (EGF) on copy number and gene expression ... 58
3.6.1 Seeding of fibroblast and NIKS for growth assay with different EGF concentrations ... 58
3.6.2 Harvesting and counting NIKS and NIKS HPV 16 cell lines ... 59
3.6.3 Extraction of total RNA ... 59
3.6.4 Preparation of cDNA ... 59
3.6.5 Quantitative real time PCR (qPCR) ... 60
3.6.6 Determination of copy number and gene expression ... 61
4. RESULTS ... 63
4.1 Phase 1 Results: Evaluation of socio-demographic characteristics ... 63
4.1.1 Geographical distribution ... 63
4.1.2 Age and marital status ... 64
4.1.3 Education and Employment status ... 66
4.1.4 Smoking and STD history ... 66
4.2 Results of Phase 2: Pelvic examination and Screening test ... 68
4.3 Phase 3 Results: HPV Screening and genotyping ... 72
4.3.1 HPV-16 and HPV-18 screening PCR ... 73
4.4 Results of phase 4: DNA sequencing ... 74
4.4.1 HPV 16 ... 74
4.4.2 HPV 35 ... 79
4.4.3 HPV18 ... 79

4.4.4 HPV56 79
4.4.5 HPV59 79
4.4.6 HPV 67 79
4.4.7 HPV 73 79
4.5 Result of phase 5: Keratinocyte and fibroblast monolayer cell culture 80
4.6 Result of phase 6: Monolayer growth assay for determining effect of epidermal growth factor (EGF) on copy number and gene expression 85
4.6.1 Increase in concentration of epidermal growth factor results in increase HPV 16 copy number 85
4.6.2 Increasing EGF concentration does not produce a proliferative effect in monolayer cultures 91
4.6.3 NIKS-HPV16 cells exposed to high concentration of EGF show variations in gene expression 93
5. Discussion 97
6. Conclusions 106
7. Future prospects 107
8. References 108

LIST OF TABLES

Table 3.1: List of primers.. **Error! Bookmark not defined.**

Table 3.2: Reagents and Buffers............................. **Error! Bookmark not defined.**

Table 3.3: Media and Supplements......................... **Error! Bookmark not defined.**

Table 3.4: Primers used in real time PCR............... **Error! Bookmark not defined.**

Table 3.5: Real time PCR cocktail.......................... **Error! Bookmark not defined.**

Table 3.6: qPCR parameters .... **Error! Bookmark not defined.**

Table 3.7: Primer used for RT-PCR........................ **Error! Bookmark not defined.**

Table 3.8: Primers used in qPCR to determine gene expression. 61

Table 4.1: Social Demographic characteristics of the females who participated in screening study... 64

Table 4.2: Knowledge about age, education and employment of the females and their respective husbands. 67

Table 4.3: Visual inspection with acetic acid findings. 71

Table 4.4: Age distribution of the 118 patients with positive VIA and pap smear.. 71

Table 4.5: Frequency distrbution of HPV 16 and 18 on basis of histopathological diagnosis 72

Table 4.6: Frequency distribution of HPV in cervical carcinoma samples in correlation with age... **Error! Bookmark not defined.**

Table 4.7: NIKS-HPV16 cells exposed to high concentration of EGF show variations in the splicing pattern of mRNA. .... **Error! Bookmark not defined.**

LIST OF FIGURES

Figure 2.1: Estimated cervical cancer incidence worldwide, 2012 Source: .....**Error! Bookmark not defined.**
Figure 2.2: Cervical cancer mortality in Pakistan across Southern Asia (https://hpvcentre.net/statistics/reports/PAK.pdf)... 10
Figure 2.3: Evolutionary tree showing the appearance of an ancestral papillomavirus between the branch point leads to amphibian and reptiles respectively (Doorbar et al., 2015). .... **Error! Bookmark not defined.**
Figure 2.4: Diagram showing evolutionary relationship among human papilloma virus. Red text indicates high risk HPV types which are confirmed carcinogens. Tropism and pathogenesis of each HPV type is also shown. Adopted from (Doorbar *et al.,* 2012). 14
Figure 2.5: Negatively stained electron micrograph of human papillomavirus showing individual capsomeres in the capsid (Chen Xiaojiang S *et al.,* 2000). **Error! Bookmark not defined.**
Figure 2.6: Schematic representation of double stranded circular genome of HPV16 (Doorbar 2006).. **Error! Bookmark not defined.**
Figure 2.7: Schematic representation of transcript map of HPV16. 18
Figure 2.8: Anatomy of the cervix (Arends *et al.,* 1998)....................................... 23
Figure 2.9: The HPV life cycle (Doorbar 2006). 26
Figure 2.10: Cervical lesion with High-grade squamous intraepithelial lesion and squamous and low grade low grade squamous intraepithelial lesion. Adopted from (Branca *et al.,* 2004).. **Error! Bookmark not defined.**
Figure 2.11: Schematic representation of Growth factor signalling pathway in human papillomavirus encompassing keratinocyte. 33
Figure 3.1: Performa used for recording socio-demographic details....................... 41
Figure 4.1: Ethnicity wise distributions of female patients. 63
Figure 4.2: Common gynaecological complaints of female patient 68
Figure 4.3: Pap smear findings of present study. 69
Figure 4.4: Microscopic photograph of normal pap smear showing isolated and clustered basal and parabasal cells. 69

Figure 4.5: Microscopic photograph of showing invasive squamous cell carcinoma of cervix of large cell keratinizing type (10X power)..70
Figure 4.6: Microscopic photograph showing high grade squamous intraepithelial lesion HSIL (CIN 2/3). There is proliferation and atypia with increased nuclei cytoplasmic ratio and loss of normal maturation (10X power). 70
Figure 4.7: Age distribution of the 118 patients with positive 72
Figure 4.8: Phylogenetic tree of HPV-16 based on partial sequencing of L1gene..76
Figure 4.9: Phylogenetic tree of HPV-16 Lineage 1 based on partial sequencing of L1gene...77
Figure 4.10: Phylogenetic tree of HPV-16 Lineage II based on partial sequencing of L1gene...78
Figure 4.11: Phylogenetic tree of HPV-18, 35, 56, 59, 67 and 73 based on partial sequencing of L1gene. 80
Figure 4.12: Microscopic images of J2 3T3 fibroblast feeder cells which serves as bed for NIKS and NIKS HPV 16 in culture medium. Images were obtained by digitally using 10x magnification objective...81
Figure 4.13: Microscopic images of NIKS harbouring HPV 16 in standard culture medium. Images were obtained by digitally using 10x magnification objective. ...81
Figure 4.14: A standard curve for GAPDH The slope (m) refers to the efficiency of the primer pairs, where a slope of -3.33 shows a perfect linear relationship between DNA copy number and Ct value and corresponds to an increase of 3.33 Ct for every 10-fold dilution .... **Error! Bookmark not defined.**
Figure 4.15: A standard curve for NIKS-HPV16. The slope (m) refers to the efficiency of the primer pairs, where a slope of -3.33 shows a perfect linear relationship between DNA copy number and Ct value and corresponds to an increase of 3.33 Ct for every 10-fold dilution........... **Error! Bookmark not defined.**
Figure 4.16: Microscopic images of monolayer culture at day 1 (24 hours),...**Error! Bookmark not defined.**
Figure 4.17: Microscopic images of monolayer culture at day 3 (48 hour), ...**Error! Bookmark not defined.**
Figure 4.18: Microscopic images of monolayer culture at day 5 (72 hour), ...**Error! Bookmark not defined.**
Figure 4.19: NIKS-HPV16 genome copy number at 72 hours compared to HPV16 genome copy number at plating.............................. **Error! Bookmark not defined.**

Figure 4.20: Evaluation of change in copy number of NIKS HPV cell 16 population. .... **Error! Bookmark not defined.**
Figure 4.21: NIKS-HPV16 monolayer growth assay. Cells were cultured for seven days in different concentration of EGF (norm=10ng/ml; +10=100ng/ml; +50= 500ng/ml) in a medium. .... **Error! Bookmark not defined.**
Figure 4.22: Quantitative real-time RT-PCR analysis of E6 transcripts in NIKS-HPV16 populations. .... **Error! Bookmark not defined.**
Figure 4.23: Quantitative real-time RT-PCR analysis of E6 transcripts in NIKS-HPV16 populations. .... **Error! Bookmark not defined.**

List of abbreviations

µL	microliter
µm	micrometer
cDNA	complementary DNA
CIN	Cervical intraepithelial lesion
cm	Centimeter
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
HSIL	High-grade squamous intraepithelial lesion
LSIL	Low-grade squamous intraepithelial lesion
M	molar
mL	milliliter
mRNA	Messenger RNA
ng	Nano gram
NIKS	Normal immortalized human keratinocytes
nm	Nano meter
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pen/strep	Penicillin/streptomycin solution
poly(A)	Polyadenylation
PV	Papillomavirus

qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-qPCR	Real-time qPcr
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TGF	Transforming growth factor
v/v	Volume/volume
VLP	Virus-like particle
w/v	Weight/volume

1. INTRODUCTION

Cancer is a disease in which there is continual unregulated proliferation of abnormal body cells. These cells have potential to metastasize to adjacent normal tissues, organs and eventually spread throughout the body. There are 277 distinct types of cancer. The most noteworthy types of cancer in males occur in the lungs, urinary bladder, colon, rectum and prostate respectively. Breast, lungs, thyroid, cervical and uterus are commonly occurring cancers in females. Blood cancer is the most prevalent cancer that affects children. It is estimated that 90 % of death due to cancer is because of metastasis (Siegel *et al.,* 2014; Topcul and Cetin 2014). Typically cells are always subject to signals that direct them to differentiate or die. Malignant cells buildup a level of autonomy from such signals, bringing about uncontrolled proliferation which may be life threatening (Hejmadi 2009).

Papillomaviruses are ubiquitous and they may infect birds, animals and humans. Hence, human papillomavirus is a broad term to refer papillomaviruses that only infect human beings (de Villiers 2013). Mostly these viruses cause warts on skin or genital area however; there are few types which have potency to cause a lesion which may progress to malignancy (Pirog *et al.,* 2014).

On basis of viral DNA sequence, more than 240 HPV types are anticipated to exist (Carvalho Nara de Oliveira *et al.,* 2010). Among them 30 types are mucosotropic and are directly involved in establishment of cervix cancer and other neoplasia of anogenital (Shukla *et al.,* 2010). Its types are cataloged as high risk human papillomavirus (HR-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and low risk human papillomavirus (LR-HPV 6,11,42 and 44) regarding their association in progressive development of lesions benign or malignant (Muñoz *et al.,* 2003). HPV 16 and 18 are ubiquitous oncogenic HR-HPV types which account for 70 % of invasive cervical cancers of female (Abreu *et al.,* 2012).

HR- HPV infects the mucosal membrane (Boda *et al.,* 2018). Basically, all cervical malignancies are caused by high risk HPV types. However, these viruses may cause oropharyngeal, penile, anal, vaginal and vulvar cancers (Burd 2016). The International Agency for Research on Cancer (IARC) divided these high risk

papillomavirus into following categories ‘carcinogenic to humans’ (Group 1), ‘probably carcinogenic to humans’ (group 2A) and “possibly carcinogenic to humans”(group 2B) (Arbyn *et al.,* 2014).

Low risk human papilloma viruses are seldom found in intraepithelial lesions, however, they may cause respiratory papillomatosis and warts in anogenital area (Workowski and Bolan 2015). Diseases caused by low risk papillomavirus results in significant morbidity and they are difficult to treat because of their reoccurrence and treatment is very expensive (Maw *et al.,* 1998; Graziottin and Serafini 2009).

HPV infection had been recognized as the major etiologic agent of carcinoma of cervix. Cervical cancer develops in the cell lining of the cervix in a female genital tract. There are pathologically distinct types of cervical cancer characterized by their location in the cervix. These are squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma. Squamous cell carcinoma is cancer of ectocervix, adenocarcinoma is cancer of endo cervix awhile adenosquamous cell carcinoma occurs in transformational zone (Stumbar *et al.,* 2019).

Individuals infected with human papillomavirus had asymptomatic course, about 1 or 2 years 90% of clearance of virus occurs. However, 10% cause persistent infection which advance in cervical carcinoma. Among this 10% of persistent infection,one-half will develop malignancy by year 30 . Many established risk factors associated with progression from human papillomavirus infection to invasive cancer include early sexual activity, multiple sex partners, smoking and use of contraceptives orally. Similarly early marriages and early age child birth are also risk factors contributing towards developing disease (De Villiers *et al.,* 2004; Oh *et al.,* 2016).

These viruses are non-enveloped have circular, double-stranded DNA tumor and size range in 55nm in diameter (Zheng Zhi-Ming and Baker 2006a; Bernard *et al.,* 2010). HPV genome has three main regions; early region encodes for viral oncogens E1, E2, E4, E5, E6 and E7 respectively. Each oncogenic protein has a specific function in viral replication and aids in evasion from immune response. L1 and L2 are virus structural proteins of Late region, while upstream regulatory region play important role in transcription and replication (Buck *et al.,* 2013; Van Doorslaer and McBride 2016).

Hence, the genome of human papillomavirus is compactly packed with *cis* regulatory components and overlapping ORFs. Transcription happens in three waves which depend on differentiation of host cell. Initial transcription started from early promotors and ended at early polyadenylation site. Intermediary transcription initiated at late promotor and terminated at early promotor and transcribed a large amount of replication proteins E1 and E2. Late transcription utilizes late promotor and late polyadenylation sites and yields increase expression of late protein L1 and L2 (Johansson and Schwartz 2013).

HPV are highly tissue tropic and limit their infection to stratified epithelium layer at specific body locales (Egawa *et al.,* 2015). Its life cycle is interrelated to host epithelium cell differentiation (Doorbar 2005). HPV genome integration into host cell is an impasse of viral life cycle. Human papillomavirus seizes its host cell replication machinery in order to replicate its own genome. Viral replication is initiated when E6 and E7 seize cell ubiquitin proteasome framework to debase retinoblastoma protein and compelling the host into S phase of cell cycle in order to use host cell replication machinery (Münger *et al.,* 1989; McBride and Warburton 2017).

Cervical cancer development comprises of four major steps including metaplastic epithelium infection at transformational zone of cervix, persistence of virus, advancement of determinedly tainted epithelium to cervix precancer and its invasion over the epithelium basement membrane (Giles and Garland 2006; Schiffman *et al.,* 2007).

The role of basal cell environment in lesion formation by human papillomavirus has been assessed, considering specifically that epithelial injury in basal membrane permits the attachment of HPV (Kines *et al.,* 2009). So as to comprehend the environmental conditions aids in viral infection, it is helpful to characterize different periods in wound healing. Basic purpose of the process is to fill the gap generated by injury and reestablish the structural stability of wounded tissue (Grossman 2013). Numerus studies had employed cell lines expressing high risk human papillomavirus. Cells infected with HPV have more proliferation and migration ability as compared to uninfected cells. (Valencia *et al.,* 2008; Yeung *et al.,* 2011). This may be due to impact of growth factor rich environment on infected cells (Cribbs *et al.,* 2002).

Keratinocyte growth is stimulated by the different factors such as epidermal growth factor (EGF), Keratinocyte growth factor (KGF), Insulin like growth factor (IGF) nerve growth factor (NGF) and fibroblast growth factors (FGF). These factors play important role in wound healing and immune response (Werner Sabine and Grose 2003a).

A pre-malignant state of the cervix (Cervical intra epithelial neoplasia) is usually asymptomatic and it can be identified by routine cytological screening tests. Frequency of disease has reduced subsequently by Papanicolaou (abbreviated as Pap) smear test in the mid-20th century. It detects any malignancy in the cervix at its early stage. Visual screening of the cervix by means of acetic acid has efficiently used in earlier detection. VIA is a simple and inexpensive test for low resource setup (Bora *et al.,* 2017).

Different studies have described various genotyping assays for human papillomavirus (HPV) (Lindh *et al.,* 2007). Most of these genotyping assays target the open reading frame L1 which is basis for phylogenetic classification of HPV. DNA sequencing is a reference technique for HPV genotyping (Briolat *et al.,* 2007). This method is beneficial for the identification of different HR-HPV and LR-HPV genotypes that could not be identified by conventional molecular methods (Meiring *et al.,* 2012).

It is a sexually transmitted disease (Stanley Margaret 2010) hence, HPV infection is essentially transmitted by skin to skin contact, for the most part yet not during sexual intercourse. The infection can occur at any age (Kjaer *et al.,* 2001). Numerous studies have revealed that developed nations had inverse relationship among age and disease prevalence. However, in low middle-income countries HPV prevalence is high over all age group. Certain cross sectional and associate investigations have demonstrated a u-shaped curve with initial peak under age 30 years and second peak among females age between 55 to 64 years old (Muñoz *et al.,* 2004; Franceschi *et al.,* 2006).

Low- or middle-income countries having limited resources and lack of screening programs and treatment facilities the major anticipated benefit of Human papillomavirus vaccination is the decrease in cervical cancer mortality. Hence, immunization and screening 1 to 3 intervals in lifetime is very useful in cervical carcinoma prevention (Goldie *et al.,* 2006).

Globally it is the fourth most prevalent malignancy in female population. Data reveals that 80 % cases occur in women from low income countries (Ferlay *et al.,* 2014). Numerous studies conducted at epidemiological, clinical and experimental level revealed that the carcinogenic potential genotypes of genital human papillomavirus (HPV) are prerequisite etiological agents for persistent infection which leads to progressive establishment of carcinoma. However, a fraction of precursor lesion develops to invasive cervical cancer (Schiffman *et al.* 2007). Its incidence is greater in Latin America, Sub Saharan Africa, central and south East Asia. Low incidence in developed states is related to early diagnosis through screening programs and management of pre-cancerous lesions (Schiffman and Solomon 2003; Parkin and Bray 2006).

Islamic Republic of Pakistan is a developing country and cervical cancer is a major health threat because of the late-stage presentation of disease. The higher mortality rate is due to absence of valuable cancer screening curriculums and vaccination programs nationwide. Social, religious taboos and stigma accompanied with the sexual transmission nature of HPV infection possibly a significant barrier in study of disease epidemiology. As a result there has been lack of documentation concerning the incidence of cervical cancer in the country (Gul *et al.,* 2015; Aziz *et al.,* 2018).

Aim and objectives

Aim:

Identification and characterization of Human papillomavirus causing cervical cancer in Pakistani women.

Objectives:

1. To evaluate sociodemographic factors influencing cervical cancer and detection of cervical lesion by screening methods; visual inspection with acetic acid (VIA) and Papanicolaou (Pap) smear among Pakistani women.
2. To determine and characterize HPV infections and to evaluate the HPV DNA in subjects with abnormal cervical cytology.
3. To identify the most prevalent type of the Human papilloma virus causing cervical cancer in Pakistani women.
4. To establish Normal Immortalized Human Keratinocytes (NIKS) or NIKS harboring HPV 16 cell population to study of the early events of epithelial wound healing *in vitro* model system and HPV16 infection.
5. To establish monolayer growth assay (1,3,5,7 days) to determine effect of epidermal growth factor (EGF) on copy number and gene expression.

2. REVIEW OF LITERATURE

2.1 Human papillomavirus and associated diseases

Human papilloma virus (HPV) is ubiquitous. It is well host-adapted virus which causes mucosal and cutaneous infection (Cubie 2013). HPV is frequently associated with the following diseases:

2.1.1 Cancer of head and neck

HPV is an etiological agent of head and neck squamous cell cancer (HNSCC) and detected in nearly 25% of all malignancies. It infects upper part of aero-digestive tract mucosal lining and causes oropharyngeal cancer (Kreimer *et al.,* 2005; Parkin and Bray 2006; Pai and Westra 2009). Incidence of HPV is higher in males as compared to females (Husain and Neyaz 2017). Risk factor include smoking, consumption of tobacco and alcohol and poor oral hygiene practice (Shaw R and Beasley 2016).

2.1.2 Anogenital warts

Several types of human papillomavirus cause genital warts. They can be sexually transmitted by skin to skin contact (Ault 2006). HPV 6 and 11 infection is frequently associated with genital warts. These are benign and have difficulty in treatment due to its reoccurrence. (Garland *et al.,* 2009; Anic *et al.,* 2011). Studies have revealed that coexistence of high risk HPV types in genital warts elevates chances to develop premalignant lesion. Furthermore, genital warts also increase risk of cervical cancer (Blomberg *et al.,* 2012; Cho *et al.,* 2017).

2.1.3 Cancer of penis

Penile cancer is a rare cancer only accounts for less than one percent of HPV can be associated with penile cancer. Basaloid as well as warty subtypes of squamous cell cancer of penis are linked with human papillomavirus infection (Stratton and Culkin 2016; Kidd *et al.,* 2017) .

Circumcision of neonates and adult can decrease risk of penile cancer (Morris *et al.,* 2011). Phimosis is a potential risk factor of penile cancer (Daling *et al.,* 2005) other

risk factors include sexual activity at early age, multiple partners , no use of condoms and poor hygiene (Morrison 2014).

2.1.4 Cancer of anus

Studies have revealed that HPV infection causes anal squamous cell carcinoma (Carter *et al.,* 2001). Human papillomavirus types 16, 18,31,33,45 are associated with anal caner (Salati and Al Kadi 2012). Anal cancer is more common in females than males (Gami *et al.,* 2014; Stier *et al.,* 2015).

2.1.5 Cancer of vulva and vagina

Majority of vaginal and vulvar cancers are associated with human papillomavirus infection (Daling *et al.,* 2002; Kang *et al.,* 2017). Squamous cell carcinoma accounts for 90% of vulvar malignancy (Bruni L *et al.,* 2015). It develops from high-grade vulvar intraepithelial neoplasia by human papillomavirus-independent differentiated type or human papillomavirus-induced usual type (Sideri *et al.,* 2005). Predominant human papillomavirus type found in vulvar neoplasia is HPV16 (Bacalbasa *et al.,* 2018). Vaginal cancer has a similar etiology as that of cancer of cervix. Risk factors of vaginal and vulvar cancer are use of tobacco, alcohol and infection with high risk HPV (Daling *et al.* 2005).

2.1.6 Cancer of cervix

Cervical cancer develops in the cell lining of the cervix in a female genital tract (Stumbar *et al.* 2019). Oncogenic human papillomavirus infection an should be recognized as the major etiologic agent of cervical cancer(De Villiers *et al.* 2004). Cancer of the cervix can be preventable, an early detection plays vital role in diagnosis and disease management (Naz *et al.,* 2018).

2.2 Incidence of cervical cancer caused by Human papillomavirus

Globally it contributes to be the fourth most prevalent malignancy in female population (Loya *et al.,* 2016) and it accounts for 530,000 new cases and 275,000 casualties/death per annum (Forman *et al.,* 2012). Data reveals that 80 % cases occur in women from low income countries (Ferlay *et al.* 2014). There is a significantly greater difference in terms of cervical cancer incidence among low to middle-income countries and high-income countries (figure 2.1). This distinction is presumably due

to introduction of efficient screening through Papanicolaou (Pap) smear, primary preventions and vaccination programs in the developed countries (Wentzensen *et al.,* 2017; Oyervides-Munoz *et al.,* 2018; Vu *et al.,* 2018). Cancer of the cervix can be preventable, an early detection plays vital role in diagnosis and disease management (Naz *et al.* 2018). In developing nations 85% of gynecological malignancies are incurable because of their disclosure at advanced stage (Zhao *et al.,* 2012).

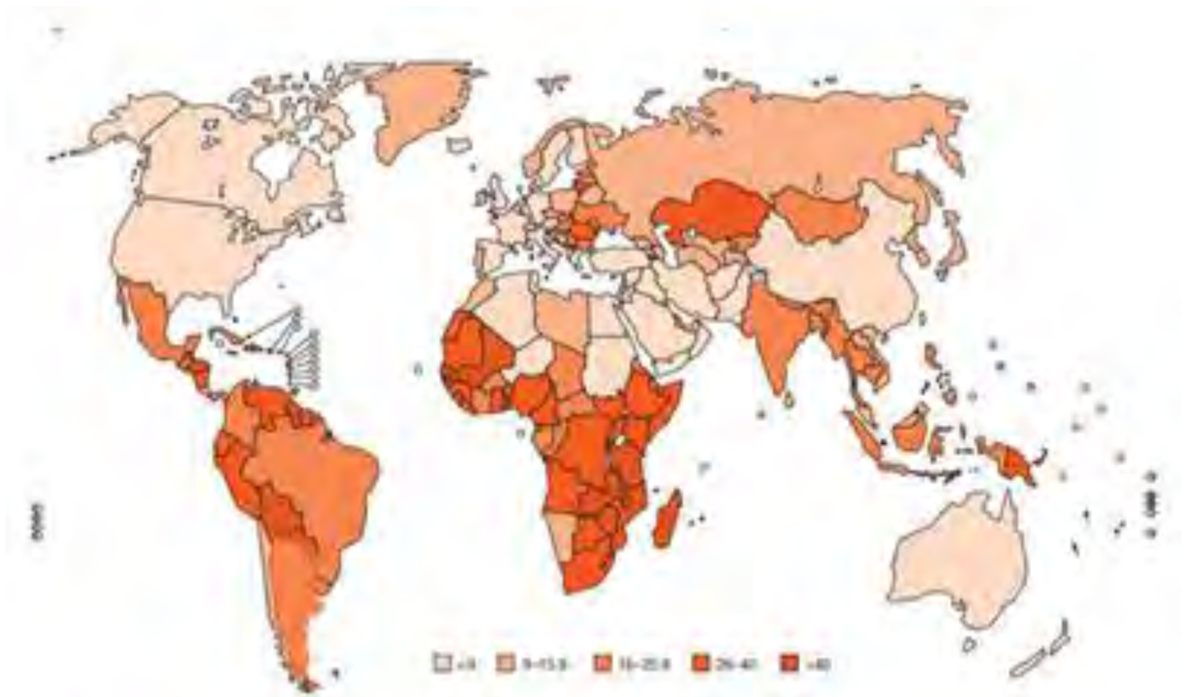


Figure 2.1: Estimated cervical cancer incidence worldwide (per 100 000) 2012
Source:

International Agency for Research on Cancer (IARC), World Health Organization (WHO). GLOBOCAN 2012: estimated cancer incidence, mortality and prevalence worldwide in 2012: cancer fact sheets:

2.2.1 Incidence of cervical cancer in Asia

Asian countries contribute to half of the global burden of cervical cancer cases annually (Garland *et al.,* 2012). In southern Asia, 116,369 accounts for new cases and 72,764 casualties per annum (Figure 2.2). The high incidence in Asia is due to lack of awareness in regard to human papillomavirus infection, screening and vaccination programs (Daniyal *et al.,* 2015).

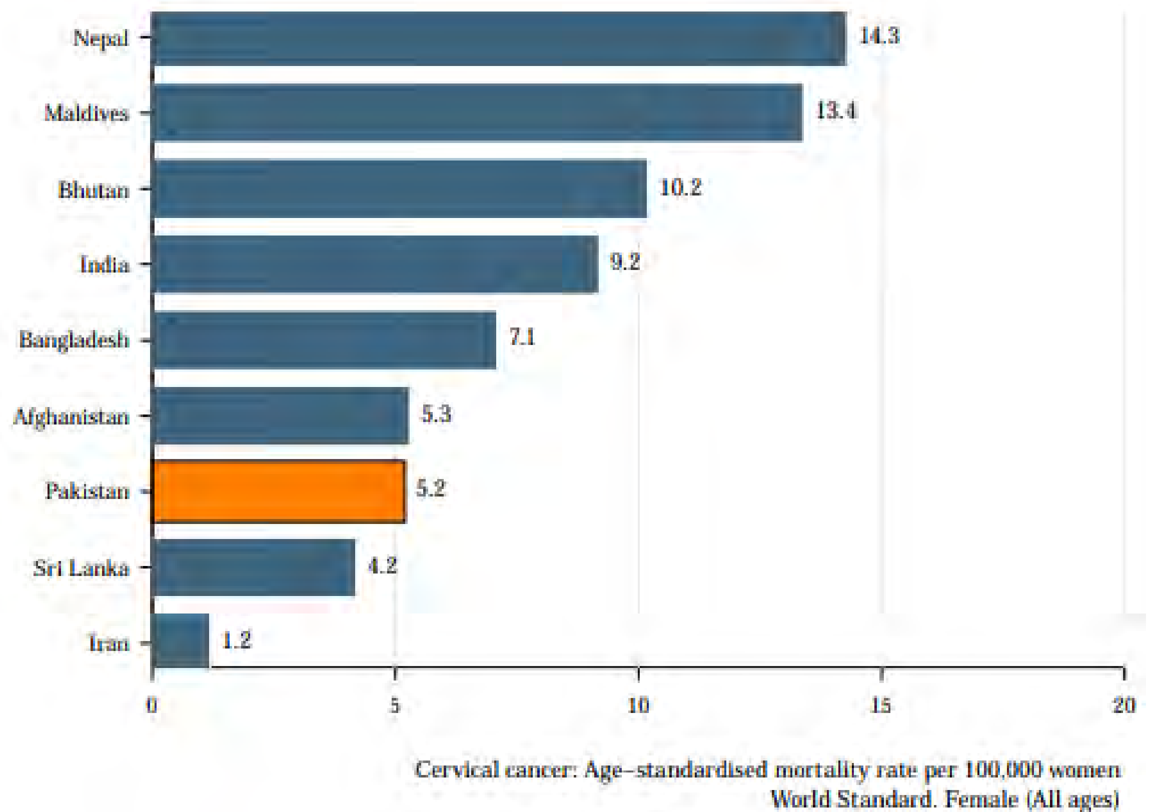


Figure 2.1: Cervical cancer mortality in Pakistan across Southern Asia (https://hpvcentre.net/statistics/reports/PAK.pdf).

2.2.2 Cervical cancer incidence in Islamic Republic of Pakistan.

The incidence of carcinoma of cervix is low in Pakistan and its late representation results in higher mortality (Gul *et al.* 2015). World Health Organization 2014 data reveals that cervical carcinoma is 3rd most common cancer in Pakistani women and its ranks as second most prevalent cancer in females aged between 15-44 years. As indicated by data on HPV Information Center in Pakistan 5233 females are diagnosed to have cervical tumor while 2876 die of this malignancy (Ferlay *et al.* 2014). In Pakistani women the HPV mode of transmission and risk factors have not been sufficiently evaluated (Khan Saeed *et al.,* 2007b) because sexually transmitted infections (STIs) are considered social and religious taboos and stigma pertaining to sex (Abdullah *et al.,* 2016).

2.3 History of discovery of human papillomaviruses (HPV)

Investigation into human papilloma virus has a long history. It is assessed that the existence of papilloma virus dates back to origin of Homo sapiens (Ho *et al.,* 1993;

Bernard *et al.,* 1994). In ancient Egypt the priests have all intellectual powers and were considered to be immediate recipient of knowledge from divine. They attribute illness and diseases such as cancer to the "will of GOD" (Javier and Butel 2008).

The primary portrayal of cervical malignancy was observed in 400 BCE by Pericles Hippocrates, a Greek Physician (Gasparini and Panatto 2009). It was viewed as serious at that time. He depicted uterine cancer as superficial ulcer which later invade uterus. In mid-19 era, the pathogenic component was perceived through a pioneer work by Italian surgeon Dr Rigoni Stern. He observed that the disease of uterine cervix was common in females living in city and once in a while happened among abstinent nuns residing in countryside. He used advanced statistical techniques to examine the reasons of death in the city between 1760-1839. He published his work under title "Fatti statistici relative alle malattie cancerose". He inferred that cervical cancer malignancy develops through sexual contact (Rigoni-Stern 1842). Later epidemiological studies revealed that cervical cancer was common in sex workers (Mak *et al.,* 2004) and it is more common in females whose husbands have multiple sex partners (Bayo *et al.,* 2002).

The first papillomavirus was detected from warts in cottontail rabbits (Shope and Hurst 1933). In 1935, Francis Peyton Rous found that cottontail rabbit papillomavirus can induce neoplasia in skin of affected rabbit. Its first finding in history that virus can cause cancer in mammals (Rous and Beard 1935). 1n 1966 he was awarded with Nobel Prize for his tumor virus work.

In1975, Harald zur Hausen described a link between HPV and cervical cancer (Zur Hausen Harald 1976). He also detected human papilloma virus from warts and cervical cancer. In 1985, Hausen and his colleagues identify the structure of human papillomavirus (Schwarz *et al.,* 1985).

Few years later his research group detected human papillomavirus type 16 in squamous cell carcinoma and human papillomavirus type 18 in adenocarcinoma in cervix (Dürst *et al.,* 1983; Boshart *et al.,* 1984). In 2008, Hausen was awarded with Nobel Prize for his remarkable work on human papillomavirus as cause of human cancer.

2.4 Classification of papillomaviruses

Papillomavirus were initially classified together with Polymaviruses in a Papovaviridae Family because the viruses shared numerous structural features (non-enveloped, double-stranded, closed circular DNA genome, an icosahedron capsid composed of 72 capsomeres), viral replication and assembly. However, major difference was the capsid and genome of papillomavirus was bigger than polyomavirus. Genome sequencing has confirmed the differences among polyomavirus and papillomavirus and there is no major homology in nucleotide or amino acid sequences. Therefore, papillomavirus were later assigned as a separate family "Papillomaviridae" by the International Committee on the Taxonomy of Viruses (De Villiers *et al.* 2004; Hoory *et al.,* 2008).

Papillomaviruses include a diverse group of viruses that can infect both animals and humans. Their origin is linked to changes in the epithelium of their ancestral host as the first reptiles emerged around at least 350 million years earlier (Bravo and Félez-Sánchez 2015). Since that time, virus have co-evolved with their respective host during speciation and little cross-transfer among species that lead to widespread distribution of viruses in birds, reptiles and mammals, yet not in amphibians or lower phylogenetic orders (Figure 2.3) (Bravo *et al.,* 2010; Thomas *et al.,* 2016). There are 240 distinct types of papillomavirus classified into 5 genera, which may possibly be considered as one of the most successful family of vertebrate viruses (Van Doorslaer *et al.,* 2016).

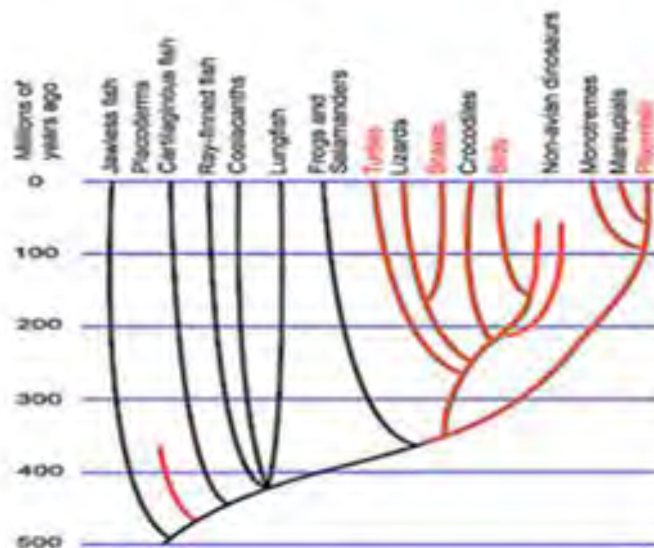


Figure 2.3: Evolutionary tree showing the appearance of an ancestral papillomavirus between the branch point leads to amphibian and reptiles respectively (Doorbar et al., 2015).

Papillomavirus classification is based on comparison of nucleotide sequence (with individual HPV being referred as genotype) instead of on serology. The L1 gene is used as the standard for papillomavirus classification, as for some PV types there is less sequence information outside of this specific region. In order to be classified as a distinct papillomavirus type, it must be at least 10% divergent from one another in their L1 nucleotide sequence arrangement. These papillomavirus "types" are assembled into large phylogenetic groups or genera, which are sorted with a Greek letter followed by a number that shows the species (De Villiers *et al*. 2004).

There are thirty different types of human papillomavirus that cause cancer of the cervix and other anogenital sites (Shukla *et al.* 2010). HPV are categorized as as high-risk human papillomavirus (HR-HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and low-risk human papillomavirus (LR-HPV 6,11,42 and 44) (Muñoz *et al.* 2003) (Figure 2.4). HPV 16 and 18 are most prevalent oncogenic high risk HPV types (Abreu *et al.* 2012).

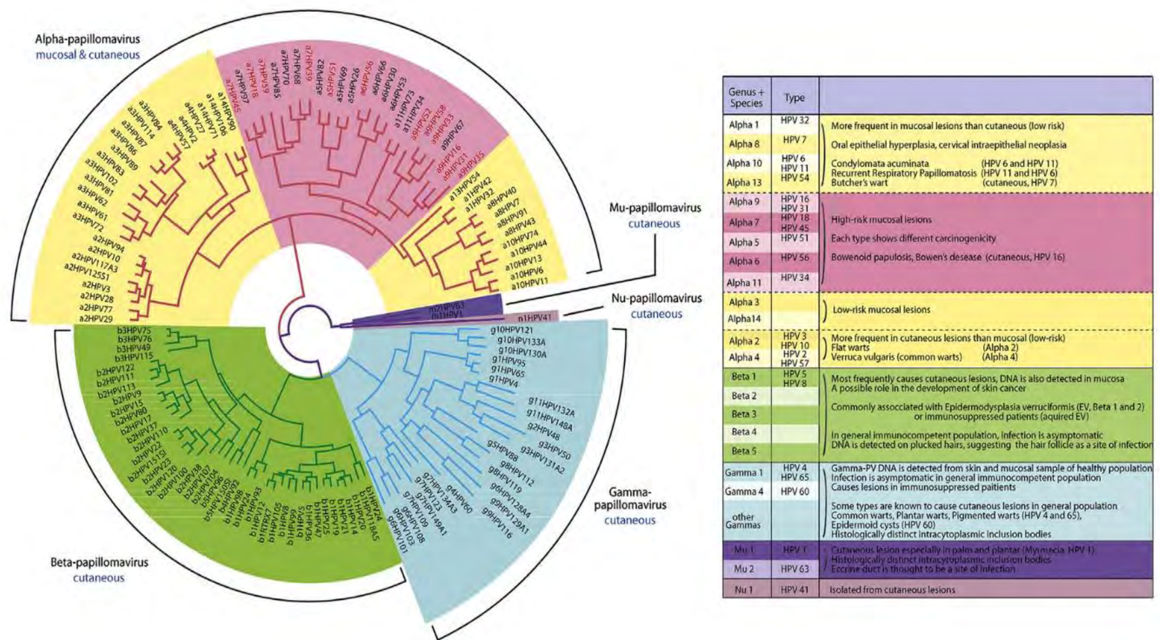


Figure 2.2: Diagram showing evolutionary relationship among human papilloma virus. Red text indicates high risk HPV types which are confirmed carcinogens. Tropism and pathogenesis of each HPV type is also shown. Adopted from (Doorbar *et al.,* 2012).

2.5 Structure of human papillomavirus

Human papillomavirus (HPV) is a relatively small non-enveloped DNA virus that is approximately 50-60nm in diameter. It consists of circular double-stranded DNA molecule (8000 base-pairs approximately) associated with histone like proteins and enclosed in an icosahedron protein capsid (Figure 2.5). Fine structure mapping reveals that virus protein coat comprise of 360 molecules of L1 protein which arranged into 72 capsomeres (Schiller and Lowy 2012). Each capsomeres is composed of five monomeric (55kDA in size) units which join to form a pentamere. The L1 pentamers are distributed in such a way forming a network of intra and inter-pentameric interaction via disulfide bonds which aids to stabilize the capsid (Modis *et al.,* 2002; Wolf *et al.,* 2010).

L2 is the secondary component of human papillomavirus capsid with about 75kDa in size and are not entirely exposed on the surface of virus. HPV contain a variable number of L2 molecules.(Kines *et al.* 2009).

Figure 2.5: Negatively stained electron micrograph of human papillomavirus showing individual capsomeres in the capsid (Chen Xiaojiang S *et al.*, 2000).

2.6 Genome of Human Papillomavirus

The structure and function of the genome of human papillomavirus is well-conserved in the entire Papillomaviridae (De Villiers *et al.* 2004). The viral genome is a circular double-stranded DNA consisted of three parts and these regions are separated by two polyadenylation poly (A) sites, which are designated as early poly (A) and late poly (A) as shown in figure 2.6 : (1) an early gene region is ~4kb, symbolized as 'E' comprised of six open reading frames (ORF) E1, E2, E4-E7). Some additional early ORFs (E3, E5 and E8) have also been identified; however, their expression is not yet equably determined throughout the PV family. (2) A late region which is ~3kb, symbolize as 'L' consist of L1 and L2 genes. (3) A long control region which is ~1kb (LCR) or upstream regulation region (URR), which lies upstream of both the early poly (A) and late poly (A) regions (Terai and Burk 2002; de Villiers 2013; Johansson and Schwartz 2013).

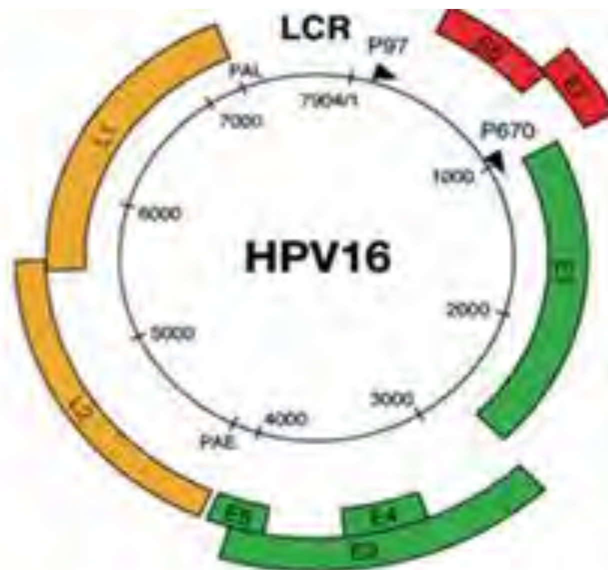


Figure 2.6: Schematic representation of double stranded circular genome of HPV16 (Doorbar 2006).

The early and late polyadenylation sites are indicated as A_E and A_L. p97 and p670 indicate the position of the early and late promoters. The two major promoters that regulate the viral gene expression in the epithelium are the 'early' p97 promoter and the 'late' p670 differentiation-dependent promoter. The p97 promoter controls the expression of early genes E6 and E7, and the p670 promoter, located within the E7 ORF, controls the expression of several early genes including E1, E2, E4 and E5 as well as late genes L1 and L2 (Smotkin and Wettstein 1986; Grassmann K *et al.*, 1996b).

Early genes code for nonstructural proteins that are essential for viral replication, cellular transformation, and proliferation. L1 and L2 encode the major and minor capsid structural proteins. The LCR has DNA recognition sites for both viral and host transcription factors and controls early gene transcription, viral amplification and cellular tropism.(McBride 2013; Egawa *et al.* 2015).

2.7 HPV gene expression

2.7.1 HPV gene transcription

Transcription is the primary step of gene expression control which is followed by regulation of mRNA and additional post-transcriptional events (Bernard 2013). Transcription of the HPV genome initiates from single DNA strand which started

from multiple promoter sites and polycistronic; it yields multiple mRNA with various ORF (Kozak 1991).The study of HPV transcripts has been done typically in W12 cell line. These cells are basically keratinocytes isolated from low-grade squamous intraepithelial cervical lesion containing HPV16 episomes. Because of the episomal nature of the viral genomes, these cells are considered to be significant for studying viral transcripts as compared to cervical cancer cell lines (CaSki and SiHa) which only contain integrated genomes and do not facilitate the viral life cycle (Stanley Margaret A *et al.,* 1989; Milligan *et al.,* 2007).

Starting point for transcription in human papilloma virus genome is variable, and depends on HPV type. Transcription starts at one point in clockwise direction at single viral DNA strand as shown in figure 2.7 .There are two major virus promoters that have been well described in alpha papillomavirus transcription; (1) "p97" the early promoter, in the virus long control region that constitutively plays an active role in the replication cycle. (2) "p670" a late differentiation-regulated promoter in the E7 ORF (Spink and Laimins 2005; Zheng Zhi-Ming and Baker 2006a). The p97 promoter is controlled by E2 virus protein and cellular transcription factors (Carson and Khan 2006).

It is reported that throughout viral life cycle transcription from early promotor remains constant. Late promotor p670 regulates transcription in certain time span and it requires activation of cellular differentiation. Late promotor activation in addition to early promotor results in increase E2 transcript level. This causes an inhibition of p97 promoter E6 and E7 expression is abrogated and pushes cells for terminal differentiation. Hence, this aids to complete viral life cycle. (Thierry 2009; Johansson and Schwartz 2013).

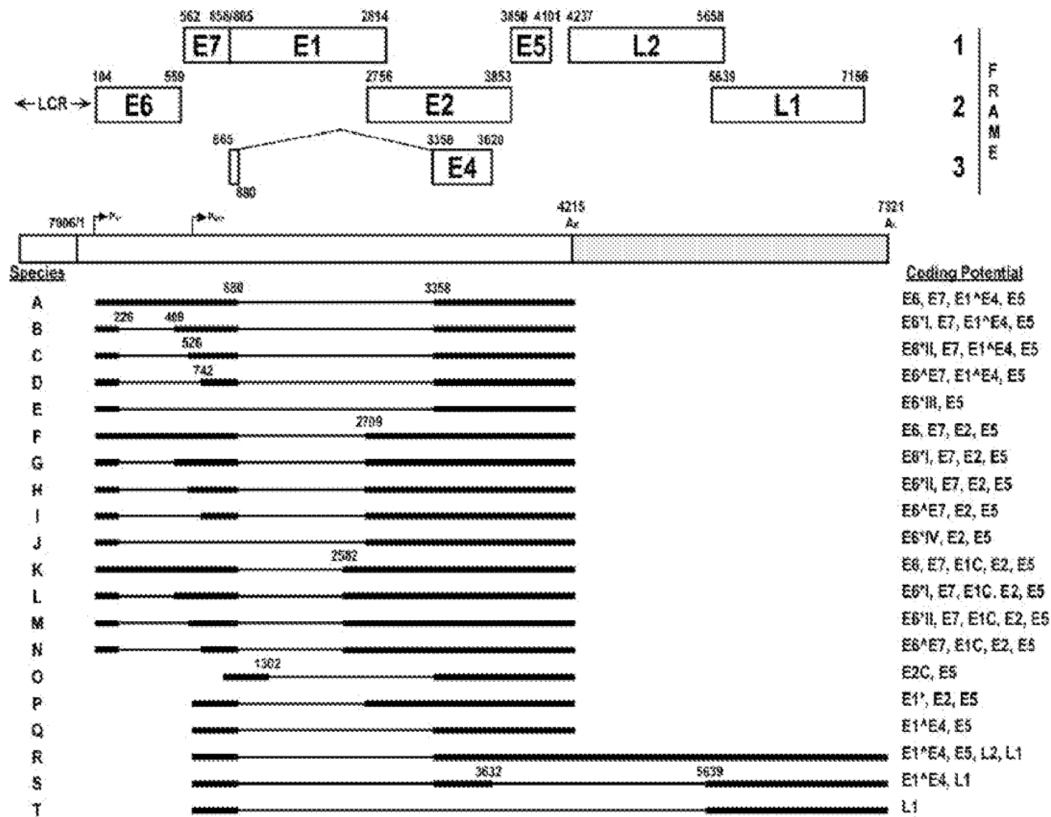


Figure 2.3: Schematic representation of transcript map of HPV16.

HPV 16 genome open reading frames are indicted as boxes and early and late promotor are underneath boxes. Direction of transcription is indicated by arrow. Nucleotide 4215 and 7321 indicates early and late polyadenylation site. Numbering on top of open reading frame indicates start and stop codon. The messenger RNA is labelled from A-T. A light line signifies introns while a dark line signifies exons respectively (Figure 2.7)(Zheng and Baker, 2006).

Human papilloma virus E6 and E7 oncogene alternative splicing transcribed by p97 promotor give rise to multiple products that may differ in functions. The messenger RNA of E6 has one intron, on splicing at 3' yields three transcripts E6, E7, E6*I and E6*II respectively (Zheng Zhi-Ming and Baker 2006a). E6*I is surplus E6 that is found in cervical carcinoma cell lines. E6*I play important role in life cycle of virus (Cornelissen et al., 1990). One of interesting fact is that splicing of E6 initiates translation of E7 (Tang *et al.,* 2006).

Transcription of full length E6 is along full-length E7. Messenger RNA product resulting from p670 are L1, L2, E1,E4, E2, and E5 respectively. L1 and E1^E4 transcripts were detected in differentiating cells of suprabasal, however, L1 transcript also detected from undifferentiated cells. This indicates that late genes transcription of genes can happen in early stage of human papillomavirus life cycle (Middleton *et al.,* 2003; Milligan *et al.* 2007; Doorbar *et al.,* 2012).

2.7.2 Translation in HPV mRNAs

Human papillomavirus transcripts translation happens with less efficiency as compared to translation of transcripts of host that takes place constitutively (Zheng and Baker, 2006).

The cellular transcripts are monocistronic while HPV transcripts are usually bicistronic or polycistronic (Firth and Brierley 2012). One of major reasons behind viral transcript low translational rate is need of rare codons necessary for translation of viral messenger RNA. Hence low stability of HPV messenger RNA and intricate translation initiation may affect translation (Zheng Zhi-Ming and Baker 2006a; Chen and Riggs 2011).

2.8 Risk factors of HPV infection and cervical cancer

Several epidemiological studies have revealed that there are varieties of risk factors associated with cervical cancer caused by HPV. These may vary in developed and undeveloped countries depending on culture, beliefs, society and socio-economic differences (Del Río-Ospina *et al.,* 2016). Risk factors for HPV infection and cervical cancer development are as follows

2.8.1 Sexually transmitted infection

Oncogenic human papillomavirus infection has been recognized as the major etiologic agent for cervical pre-cancerous and cancerous lesion. HPV infection is usually transmitted by sexual activity and causes cervical intraepithelial lesions. Persistent infection with High risk HPV 16 and HPV 18 types develop into cervical malignancy (Bruni Laia *et al.,* 2010; Wardak 2016).

2.8.2 Age

The peak age to develop HPV infection in the early 20s. Females less than 20 years old rarely develop cervical cancer, however, risk increases at age around 30s. Many studies have documented that the peak age to develop cervical cancer is between 40 to 50 years of age. However, its precursor lesion develops earlier (5-10 years) (Winer *et al.,* 2005; Moscicki *et al.,* 2010; Saraiya *et al.,* 2013).

2.8.3 Early Marriage and pregnancy

Previous data showed that marriage and birth of child at a very early age could influence the risk of HPV infection and cervix carcinogenesis. This susceptibility may be due to steroidal hormone influence on human papillomavirus infection, host immune response to HPV infection in puberty and biological immaturity during adolescence (Elson *et al.,* 2000; Louie *et al.,* 2009).

2.8.4 Parity

Parity is an important co-factor in developing cervical cancer in females with HPV infection. It has been reported that, the transformational zone has direct exposure to human papillomavirus in multiparous female (Autier *et al.,* 1996; Jensen *et al.,* 2013). This risk factor increases with women who have more than three or four children as compared to those who have none or one (Muñoz *et al.,* 2002).

2.8.5 Multiple sexual partners

Having multiple sex partners and unprotected sexual activity is potential factor to develop cervical cancer. This factor is usually attributable to high risk of HPV infection. Epidemiological studies show that having many sexual partners, who may or may not have HPV infection, is still an independent risk to develop cervical carcinoma. (Castellsagué *et al.,* 2003; Liu *et al.,* 2015).

2.8.6 Impaired immune system

The immune system plays a vital role in the steps from HPV infection to carcinogenesis. Females with compromised immune response have increased risk of developing infection and malignancy (Nunes *et al.,* 2018). Different immune cells are involved at different stages of disease progression. High risk HPV induces several

modifications to establish persistent infection and disease progression(Song *et al.,* 2015)

The increase in HPV prevalence in older age groups could be attributed to human estrogen and progesterone level changes affecting the cervical epithelial metaplasia process and support the HPV replication; on the other hand, the decline of the body's immune function leads to decreased ability to clear HPV and increases the susceptibility to an HPV infection (Zhou *et al.,* 2017).

2.8.7 Use of oral contraceptives

Use of oral contraceptive should be risk factor associated with cancer of cervix. It is suggested that use of oral contraceptive for more than five years may increase risk to develop cancer. Furthermore, incidence of malignancy is higher in persons who have used injectable progesterone for more than five years (Cogliano *et al.,* 2005; Cancer 2007).

2.8.8 Smoking

International Agency for Research on Cancer (IARC) has classified Smoking or tobacco use is an important risk factor for cervical carcinoma (Walboomers *et al.,* 1999). Several studies have demonstrated association between smoking history or current smoking and carcinogenesis (Hildesheim *et al.,* 2001; Kim *et al.,* 2012). Females who smoke are at double the risk of developing cervical malignancy as compared to those who don't. However, passive smoking is not linked with higher risk of cervical cancer(Roura *et al.,* 2014).

2.8.9 Socio-economic factors

Socio-economic status is linked with cancer incidence, mortality and morbidity (Hastert *et al.,* 2015). Studies have revealed that poor socioeconomic status, lack of education and poor hygiene has positive association with progression of disease (Ali *et al.,* 2010; Das *et al.,* 2013; Jbeen *et al.,* 2013). Hence, higher level of education is positively associated with better knowledge about cervical cancer screening (Aldohaian *et al.,* 2019a). Person with low socio economic status face several economic barriers, lack of information and access to health care institutes (Dunlop *et al.,* 2000).

2.9 HPV infection transmission

HPV virus may be transmitted among humans in the following ways:

2.9.1 Horizontal transmission

Alpha-human papillomavirus has the affinity to cause oral and anogenital mucosa infection, while Beta and Gamma papillomaviruses infects the skin. Sexual activity is the most common mode of horizontal transmission of anogenital HPV via contact with infected penile, vaginal, vulvar, cervical, or anal epithelial surface (Forman *et al.* 2012; Satterwhite *et al.,* 2013). HPV can easily transmit by sexual contact with an infected partner presumably through microscopic abrasion in the skin or mucosa (Kjaer *et al.* 2001).

HPV transmission can take place from one anogenital site to other by self-inoculation (Widdice *et al.,* 2013). Identification of HPV infection transmission by hand carriage indicates HPV transmission by digital–genital contact (Winer *et al.,* 2010; Sabeena *et al.,* 2017). A few studies have reported that HPV non-sexual transmission is not common. However, numerous studies demonstrate that HPV infection can be transmitted other than sexual activity. The possible way of non-sexual transmission is fomites (Ryndock and Meyers 2014; Sabeena *et al.* 2017). HPV infection can be easily transmitted. However, the degree of infection transmission varies among HPV type and across population (Rozendaal *et al.,* 2000; hui Xu *et al.,* 2017).

2.9.2 Vertical Transmission

HPV infection vertical transmission can occur in following three ways: (1) The periconceptual transmission (during fertilization or immediately after fertilization), (2) prenatal transmission (in pregnancy), and (3) perinatal transmission (during or immediately after birth) (Tenti *et al.,* 1999; Sarkola *et al.,* 2008; Foresta *et al.,* 2011). Vertical transmission of HPV from mother to child was first suggested in early 1950 (Smith Elaine M *et al.,* 2010). A Few cases of anogenital warts in newborns have been reported. HPV DNA has also detected in mucosal scrapings obtained from infants (Cason and Mant 2005; LaCour and Trimble 2012).

2.10 Physiology of cervix uterine

Infection in the female genital tract not harms the individual's health but it has adverse effect on her fertility and pregnancy. Biological and epidemiological studies have firmly confirmed the role of high-risk human papillomavirus in cervical cancer (Zekan *et al.,* 2014; Zhang *et al.,* 2017). It can infect any tissue of the lower genital tract in females (Munoz *et al.,* 2006). It is important to understand the physiology of the cervix to know how this specific anatomical region is vulnerable to papillomavirus related infections. The part of lower uterus is cervix and it connects to vagina by means of cervix external and internal opening as shown in figure 2.8.

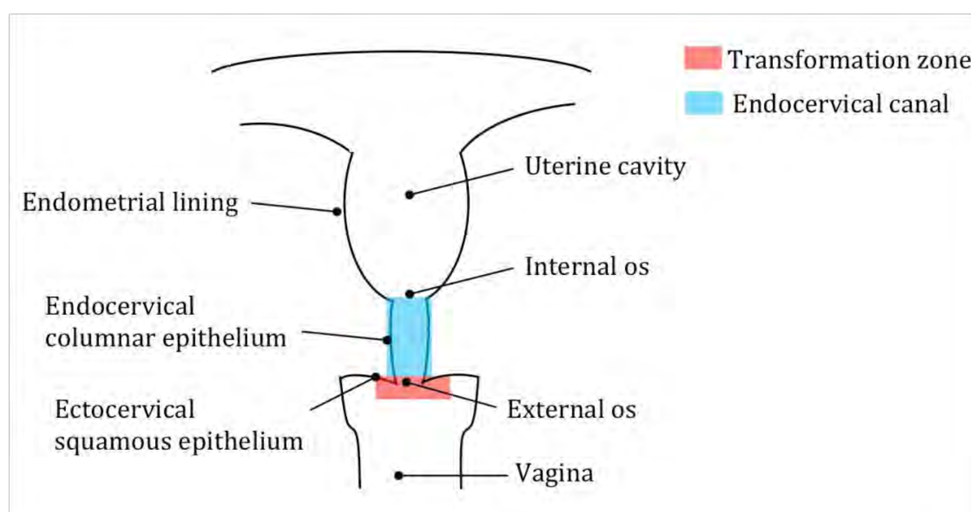


Figure 2.4: Anatomy of the cervix (Arends *et al.,* 1998).

The cervical surface is composed of two types of epithelium. The endocervical canal is in the center of cervix and is lined by mucus-secreting columnar epithelium. The ectocervix is vaginal side of the cervix is made of squamous epithelium. The Squamous columnar junction is the point where endocervical columnar epithelium meets ectocervical squamous epithelium. The transformation zone (TZ) is site of metaplastic tissue among columnar tissue and squamous epithelium. It is particular site susceptible to infection and it is site where lesions are detected (Adekunle 2012; Herfs *et al.,* 2012).

2.11 Life cycle of Human Papilloma virus

The HPV life cycle has two main events early and late. Viral infection and episome establishment in nucleus of host cell are main stages of early events. However,

genome amplification, capsid structural protein expression and new virus assembly and release occur in late events.

2.11.1 Early Events

2.11.1.1 Infection of the basal layer of the epithelium

Human papillomavirus are extremely an epitheliotropic (Castillo 2013). The life cycle of HPV is linked to differentiation of infected epithelial cell (Kajitani *et al.,* 2012). Epithelial basal cells are the target cells for HPV infection. In order to establish an infection HPV invade basal cells of the epithelium through cellular abrasions or minor trauma. The cervical transformation zone is highly prone to HPV infection because the basal layer of epithelium being easily accessible to the virus because of low immune surveillance (Doorbar 2006; Stanley Margaret A 2012).

Inside a typical epithelium, basal layer has cells which are mitotically active. Generally, there are two kinds of cells inside the basal and parabasal layers, transit amplifying cells and epidermal stem cells. Basal layer stem cells after division leave their compartment to enter the parabasal layer and form transit amplifying cells. These daughter stem cells can then undergo cell divisions before leaving cell cycle and compelling to the cellular terminal differentiation program. Hence, in order to establish a persistent infection virus must gain entry to stem cells (Fuchs *et al.,* 2004; Kolly *et al.,* 2005).

The proposed mechanism of Human papillomavirus infection implicates the L1 and L2 capsid proteins in the process (Schiller *et al.,* 2010). L1 binds heparan sulfate proteoglycans located on basement membranes that become accessible to the virus via micro wounds in the tissue. This prompts the cleavage of L2 by furin proteins, which thus permits L1 to tie to alpha-6 integrin receptors present on keratinocytes surface (Joyce *et al.,* 1999; Richards *et al.,* 2006). Subsequently there is internalization of virus by endocytosis, it may involve clathrin- and cavelolae-mediated pathways depending upon the specific HPV type (Bousarghin *et al.,* 2003).

2.11.1.2 Establishment and maintenance of HPV episomes in in the lower epithelial layers

After establishing infection, virus uncoats itself and establishes viral genome as episome in cellular nucleus. Primarily, virus undergoes a transient replication before replicating cellular DNA in order to increase its own copy number (Kadaja *et al.,* 2009)

2.11.1.3 Role of Early proteins

E1 and E2 are viral replication proteins, expressed from p97 promotor play important role in early amplification phase (Figure 2.9). E1 is DNA helicase involve in viral genome replication, they might be superfluous for episome maintenance-replication after copy number has stabilized (Egawa *et al.,* 2012). However, E2 is important in genome partitioning and it also regulate viral transcription (Maglennon *et al.,* 2011). E2 viral protein has many binding sites in viral LCR. In process of viral replication E2 can recruits the viral E1 helicase into specific E1 binding motifs. It is suggested that there is difference in E1 replication helicase and cellular replication helicases, which allows disconnection of the viral replication from cellular replication in genome establishment and amplification process (McBride 2013; Bergvall *et al.,* 2016).

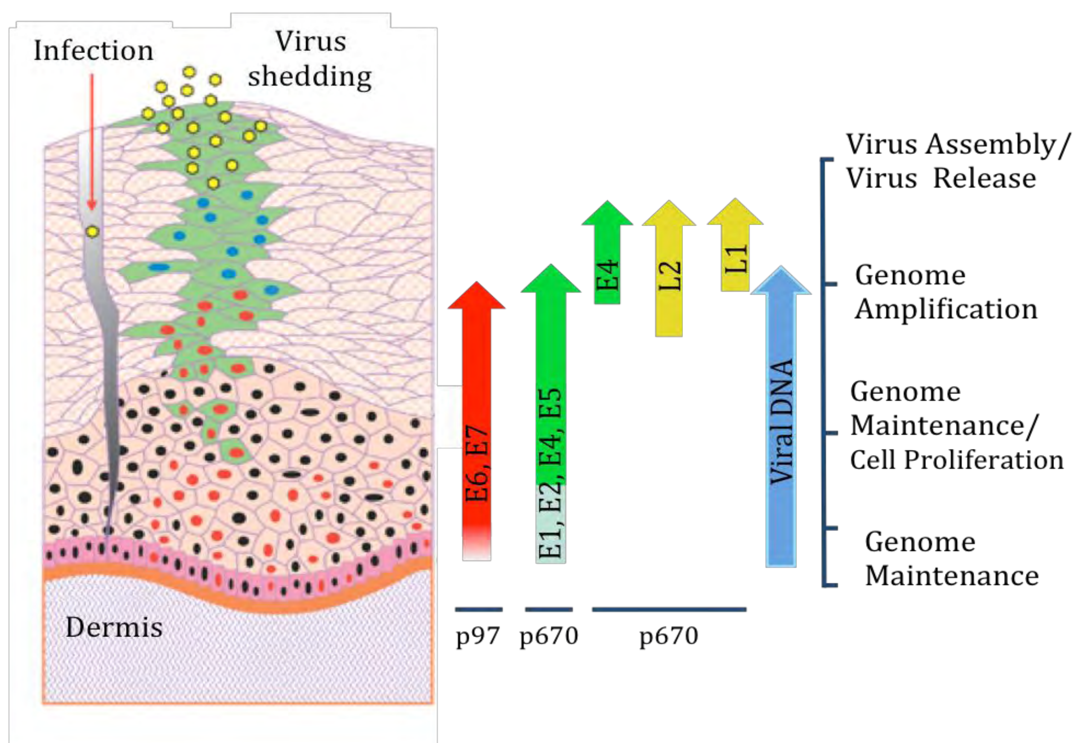


Figure 2.5: The HPV life cycle (Doorbar 2006).

2.11.1.4 Cell proliferation

As basal cells are only mitotically active cells in uninfected epithelium, furthermore, when they are pushed out of the basal layer, they leave cell cycle and undergo terminal differentiation. This differentiation is detrimental to virus, as it depends on

host replication machinery. therefore virus in infected epithelium brings delay in onset of differentiation by viral E6 and E7 proteins, and push cells into S phase even in suprabasal layer (Doorbar 2006). E7 bind and degrade Rb protein which release E2 and cause transition in S phase of cell cycle (Fan *et al.,* 2018). E6 binds and degrade p53 protein to inhibit cell death (Howie *et al.,* 2009). These E6 and E7 functional differences help in determining disease pathogenesis among HPV types (White and Howley 2013).

2.11.2 Late Events

2.11.2.1 HPV Genome amplification and maintenance in the upper epithelium

E6 and E7 protein expression allows the infected cell in upper epithelial layer to re-enter in S-phase in order to increase viral copy number (Roman 2006). There is additionally a requirement for the E1 and E2 proteins for upregulation of differentiation dependent promotor (p670) which is necessary for amplification of genome. In genome amplification, E4 and E5 viral protein contribute indirectly by modifying cell environment (Bodily and Laimins 2011). E4 protein prevent mitotic entry in G2 phase by arresting cells (Davy *et al.,* 2005). E5 viral protein has ability to interfere apoptosis and intracellular trafficking of endo-cytotic vesicles. E5 play major role in genome amplification by stabilizing EGFR and it also enhance MAP kinase activity and EGF signaling (Fehrmann *et al.,* 2003; Genther *et al.,* 2003).

2.11.2.2 Virus assembly and release

Human papilloma virus life cycle completion eventually involves L1 (minor coat protein) expression to exit cell from cell cycle and L2 (major coat protein) to permit packaging of genome (Doorbar 2006). The process of genome encapsidation involves L2 and it is enhanced by E2 viral protein (Johansson *et al.,* 2012). E4 viral protein aids in virus release by disrupting cellular keratin network and it also make epithelial layer very fragile (Wang *et al.,* 2004; McIntosh *et al.,* 2010).

Maturation of virus happens in superficial dying keratinocytes. Prior to virus release these keratinocytes lose their mitochondrial oxidative phosphorylation and change from a reducing to an oxidizing environment. This enables aggregation of di-sulphide bonds among L1 viral proteins which results into extreme stable viral infection (Finnen *et al.,* 2003).

2.11.2.3 Abortive infections

Human papillomavirus causes both low and high-grade intraepithelial (LSIL and HSIL) diseases. Usually LSIL establishes a productive human papillomavirus infection as in such case new infectious HPV are produced (Doorbar *et al.* 2012).Whereas, there are some sites like transformational zone where productive HPV infection is not efficiently supported and virus causes abortive infection (HSIL)(Doorbar 2006). In HSIL, HPV do not replicate and it leads to progressive cancer if left untreated. Once lesion become high grade cellular differentiation is delayed which is associated with high levels of E6 and E7 viral proteins and less expression of late proteins (E4,L1 and L2)(Middleton *et al.* 2003; Hu *et al.,* 2007).

2.12 Pathology grading of Human papilloma virus lesion

Development of cervical malignancy from a precursor lesion to a progressive invasive cancer involves series of well-defined neoplastic stages. The cervical smear reporting system is has evolved over time, basically there are two types of histopathological grading systems to classify lesion. These are the cervical intraepithelial neoplasia (CIN) system and the Bethesda system (Burd 2003).

In 1973, CIN system of classification was introduced the idea of cervical intraepithelial neoplasia as disease continuum. In this system, CIN 1 is mild dysplasia i.e. one third of epithelium has shown dysplasia, CIN2 refers to moderate dysplasia which means two third of epithelium has dysplasia, while CIN 3 is chronic dysplasia and cancer i.e. full epithelium is dysplasia as shown in figure 2.10 (Richart 1973; Broder 1992).

The Bethesda system was introduced in 1988 for advanced understanding of neoplastic lesions. Bethesda system classifies abnormalities in squamous cells in four possible ways: atypical squamous cell (ASC), low grade squamous intraepithelial lesion (LSIL), High grade squamous intraepithelial lesion (HSIL) and cervical carcinoma. An integral part of this reporting system is a “statement of adequacy”. The Bethesda system is modified several times.in up dated version of classification LSIL is similar to CIN 1 while HSIL is equal to CIN2 and CIN3 as shown in figure 2.10 (Solomon D 1989; Luff *et al.,* 1992; Solomon Diane *et al.,* 2002).

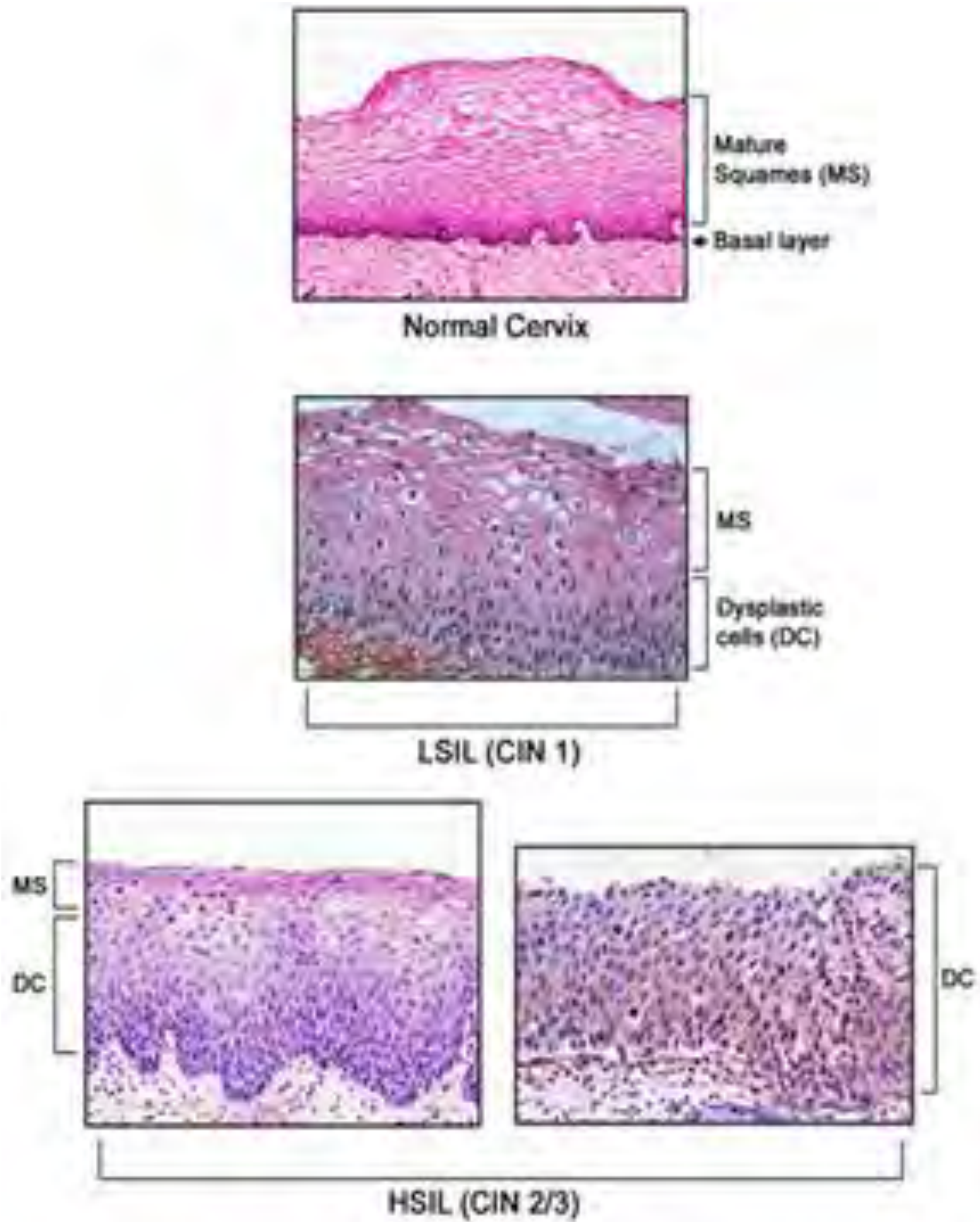


Figure 2.10: Cervical lesion with High-grade squamous intraepithelial lesion and squamous and low grade low grade squamous intraepithelial lesion. Adopted from (Branca *et al.*, 2004).

2.13 Immune response and clearance of HPV infection

The host immune response plays vital role in regression and progression of human papillomavirus infection. Spontaneous clearance of virus is result of effective immune response, however, compromised immune response leads to the disease progression (Bedoya *et al.,* 2013). Host immune system clears 90 % of virus infection within one or two years, while only 10 % advances into a persistent viral infection. Of these 10%, only one-half develop cancer via high risk infection by period the of 30 years The main reason behind viral infection persistence for many years is because virus is non-lytic and absence of inflammation at infection site (Stanley MA 2009; Deligeoroglou *et al.,* 2013).

The Innate immune system is first line of defense against HPV infection. It makes a pro-inflammatory micro-environment to eliminate virus by recruiting several immunological cells and initiate efficient acquired immune response (Egeblad *et al.,* 2010; Scott *et al.,* 2015). Furthermore, an important antiviral defense mechanism is interferon response towards human papillomavirus infection. Interferon signaling pathway is suppressed with both E6 and E7 viral proteins. (Pett *et al.,* 2006; Kanodia *et al.,* 2007). These viral protein also decrease E-cadherin level which results in correlates in reduction of Langerhans cells and reduce immune response (Laurson *et al.,* 2010).

2.14 Wound healing process

Micro-abrasion or wounding of epithelial layer facilitates human papillomavirus infection had been perceived for almost 100 years. The basic purpose of process of healing is to fill the space formed by the tissue damage and to maintain integrity of wounded tissue. The process of wound healing is intricate and it is divided into inflammatory, proliferative and remodeling phase (Shope and Hurst 1933; Eming *et al.,* 2007; Sinno and Prakash 2013).

Inflammatory phase principally serves to eradicate the injury causing agent and establish environment for wound healing. At this phase there is release of several cytokines and growth factors which draw in encompassing fibroblasts. These are interleukin-6 (IL-6), platelet derived growth factors (PDGF), fibroblast growth

factors (FGF) and transforming growth factors beta-1 (TGFβ-1) and epidermal growth factors (EGF) (Heldin and Westermark 1999; Qing 2017).

The entry of fibroblasts marks the beginning of proliferative phase in wound healing. The main purpose of this phase is to fill the injured gap by making new tissues. Collagen is synthesized and released by the recruited fibroblasts. They also release growth factors that initiate the development of blood vessels in a process called angiogenesis, which at that point advances endothelial cell multiplication and movement. The endothelial and fibroblasts cells together form a granulation tissue that serve as a base for development of wounded tissue. Epithelization is the last part of proliferative phase. It involves regeneration, propagation and differentiation of epithelium cell at wounded site to create new area same as before injury (Iruela-Arispe and Dvorak 1997; Risau 1997).

Remodeling is the final phase of the wound healing. It starts at different times usually weeks after the damage, proceeds for a half year or more. At this stage scar tissue is made by collagen synthesis. The scar tissue ends up avascular and may have accomplished 70-80% of tensile strength following three months (Levenson *et al.,* 1965; Clark *et al.,* 1996; Werner S. *et al.,* 2007).

2.14.1 Growth factors involve in wound healing

Wound healing pathway is dynamic in terms of series of events which involve cytokines, inflammatory cells and several growth factors. Growth factors are basically molecules that stimulate proliferation, differentiation or migration of cells. They may be a tiny molecule (hormone) or it may be macromolecule (protein).These growth factors are released by fibroblasts and keratinocytes which bind to receptors present on cell surface to form new epithelium. Many growth factors are versatile in there function and stimulate cell division in different type of cells. However, few of these are specific in their function in one particular cell type. Fibroblasts release some potent growth factors which are essential in proliferation. These are transforming growth factors beta (TGF-β) and fibroblast growth factors 1-18 (FGF1- FGF18) and platelet derived growth factors (PDGF). Keratinocyte derived growth factors also paly vital role in wound healing such as transforming growth factors-α (TGF-α) , transforming growth factor-β (TGF-β), epidermal growth factor, keratinocyte growth

factor (KGF),interleukin-6 (IL-6) and interleukin (IL-8) (Chesney and Bucala 2000; Abe *et al.,* 2001; Seeger and Paller 2015; Qing 2017).

2.14.2 Role of Epidermal growth factor in wound healing

EGF is a potent mitogen for cells like endothelial, epithelial and fibroblast. In 1962, Stanley Cohen was first to isolate it from mouse submaxillary glands and awarded with Nobel Prize for his work on growth factors. It is a polypeptide consists of 53 amino acids and stimulates synthesis of fibronectin, angiogenesis and activity of collagenase. EGF plays vital role in process of wound healing. Since its discovery, development of novel therapies has incorporated the usage of EGF in wound healing treatment. Moreover, EGFR inhibitor is another therapeutic option for cancer treatment and therapies which target EGF and EGFR are useful for treating cutaneous wound and carcinomas (Cohen 1962; Bodnar 2013).

Epidermal growth factors (EGFR) expressed all through the epidermis but in basal layer the expression is more prominent. EGF binds to these specific receptors on cell surface to initiate signal transduction which aids in proliferation (Seeger and Paller 2015). EGFR can be activated in autocrine or paracrine manner. In autocrine, activation of EGFR ligands are created by keratinocyte while, in paracrine, ligands are synthesized by macrophages, platelets or fibroblast (Tokumaru *et al.,* 2005; Shirakata 2010).

EGF itself instigates migration of keratinocyte cell in *in vitro* models, it can also found in wound fluid. However, the role of EGF in keratinocyte cell migration *in vivo is* not yet recognized (Peplow and Chatterjee 2013). In wound healing, EGFR signaling is important in migration and proliferation of keratinocyte. Activation of EGFR stimulates MAPK signaling pathway and lead to growth stimulation. It also induces cell growth by signal transducer and activator of transcription signaling (STAT) (Haase *et al.,* 2003; Quesnelle *et al.,* 2007).

The remarkable role of EGF receptor in keratinocyte development and migration makes it possible target for HPV oncogenes. Transcription factor AP1 is downstream target of EGF receptor and it is essential for viral LCR regulation. E5 viral protein mainly regulates the EGFR signaling, it also prevents the degradation of EGFR and increase EGFR level at cellular surface. Detailed growth factor signaling pathway is

shown in figure 2.11 (Chong *et al.,* 1991; Pedroza-Saavedra *et al.,* 2010; Woodworth *et al.,* 2011).

It is still unclear that, in early Human papillomavirus infection rise in cell proliferation is due to expression of viral oncogenic protein or it may be because of wound healing proliferative response. Different events of wound healing in context to infection cause by human papillomavirus can be demonstrated *in vitro* by using Keratinocytes monolayer growth cultures (Doorbar *et al.,* 2015).

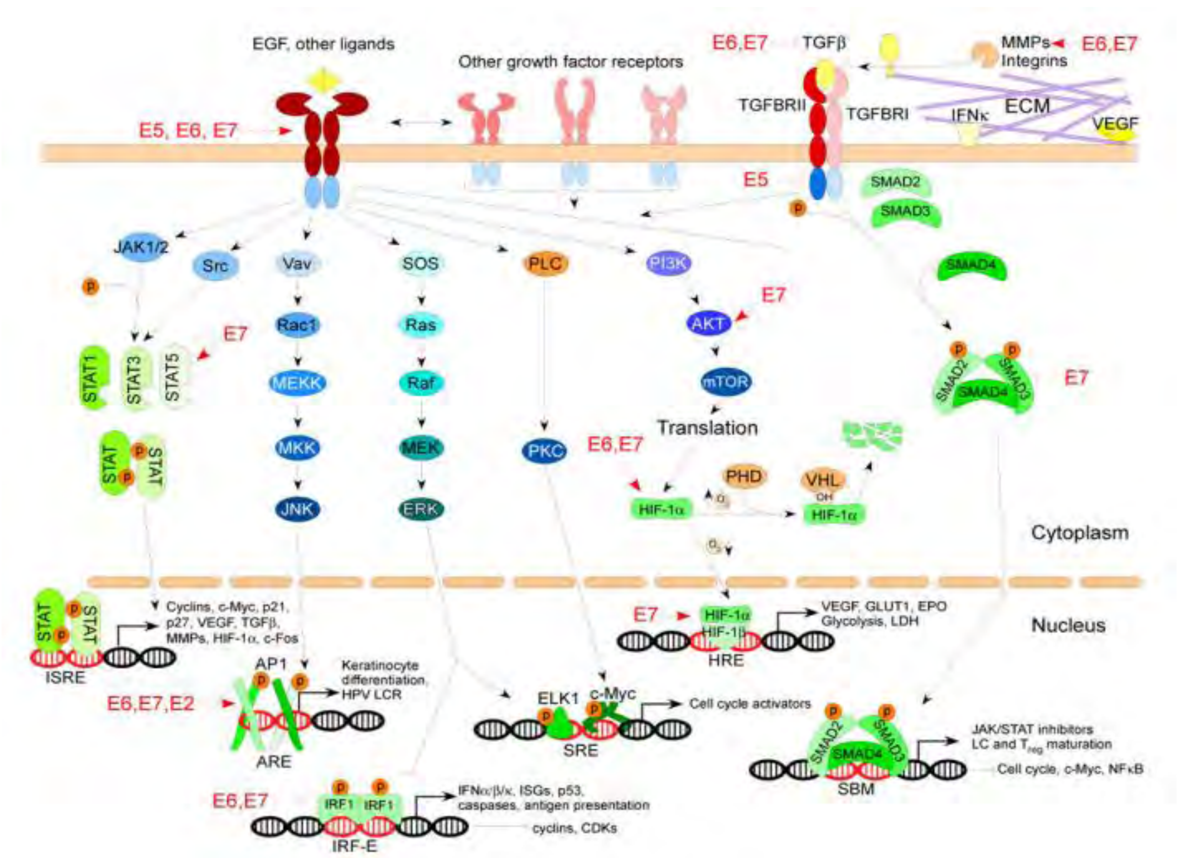


Figure 2.6: Schematic representation of Growth factor signalling pathway in human papillomavirus encompassing keratinocyte.

EGF receptor activate STAT3 and STAT5, MAPK, phospholipase C and Jun Kinase (JNK) .c-Met and PDGF activates similar pathway. Arrows and bars indicate regulation points by human papillomavirus oncogene protein. Kinases are shown in blue, Receptors (red), ligands (yellow), and transcription factor (green) respectively. ARE,API are response element, hypoxia response element (HRE), Interferon sensitive

response element (ISRE) proline hydroxylase (PHD) and binding motif (SBM/SMAD) are also shown in figure 2.11 (Woodby *et al.,* 2016).

2.15 Detection of Human papilloma virus

2.15.1 Screening of HPV associated lesion

Carcinoma of cervix is one of the cancers that can be detected at its early stage via screening. Infection cause by HPV is asymptomatic hence screening of cervical cancer is significant in early stages (Saraiya *et al.,* 2007). Screening tests use to detect HPV associated lesions are as follows:

2.15.1.1 Papanicolaou test.

Incidence of cervical cancer has reduced subsequently by the introduction of Papanicolaou (abbreviated as Pap) smear test in the mid-20th century. Papanicolaou test is named after George Nicholas Papanicolaou known as “father of cytology”. He was first to describe that cervix cells have some characteristics which aids in cancer diagnosis (Shaw Patricia A 2000). Pap smear test is primary screening method to identify any intraepithelial changes in cervix. It is an effective technique with 70% sensitivity to detect any precancerous lesion and cancer. (Ansari *et al.,* 2012; Balachandran 2012).

Cervical cells were obtain from the transformational zone of cervix by help of Ayer spatula or brush and fixed on glass slide, stained by Papanicolaou stain and examined under microscope to detect any histologic abnormality. An alternative to this conventional technique is Liquid base cytology. In this technique cervical cells is collected by help of brush and transferred in to fixative solution and spread in a monolayer (Hoda *et al.,* 2013; Sachan *et al.,* 2018). The histopathological findings are reported according to “The Bethesda system”. It is reporting system for cervical squamous intraepithelial lesions and divided into low-grade squamous intraepithelial lesion (LSIL)and High grade intraepithelial lesion (HSIL) (Nayar and Wilbur 2015). Colposcopy or biopsy is done if result of Pap test is abnormal (Gray and Kocjan 2010).

Screening test done along with human papillomavirus DNA testing favors early detection of cervical intraepithelial neoplasia (Patel *et al.,* 2011). Cervical cancer

screening is recommended for women at mid-30, as malignancy may develop at age of above 40years old, however, its precursor lesion usually develops earlier (5 to 10 years) (Maleki *et al.,* 2015a).

2.15.1.2 Visual inspection with acetic acid

Visual screening of the cervix by means of acetic acid has efficiently used in early detection. VIA (visual inspection with acetic acid) is a simple and inexpensive test in low resource setup. (Mehta *et al.,* 2013; Manisha *et al.,* 2017). In this method, 3 to 5% acetic acid is applied on cervix for one to two minutes to observe any aceto-white area on cervix(Akinola *et al.,* 2007). Colposcopy is recommended for any abnormal VIA findings (Koliopoulos *et al.,* 2007).

2.15.1.3 Immunohistochemistry

Cervical cancer histo-pathological diagnosis can be more effective by immunohistochemical staining. Cervical intraepithelial neoplasia showed increase level of cyclin dependent kinase inhibitor p16. It is suggested that staining with this marker will improve the accurate identification of cervical intraepithelial lesion in tissue specimen (Klaes *et al.,* 2002). It has been observed that HPV E7 oncogenic protein when inactivates retinoblastoma protein the expression of p16INK4a is increased (Ishikawa *et al.,* 2006). Immunohistochemistry with p16INK4a bio marker reduce false-positive/negative interpretations which are helpful in cancer diagnosis (Klaes *et al.* 2002; Carozzi *et al.,* 2013). Another important cell proliferation marker is Ki-67 (Iaconis *et al.,* 2007). P16 and Ki-67 are alternative biomarkers in detection of cervical cancer ambiguous cases (Zhong *et al.,* 2015).

2.15.2 Molecular methods for HPV detection

Human papillomavirus can be detected by screening tests based on morphological characteristics of cervical samples, however, molecular methods provide accurate detection (Shen-Gunther and Yu 2011). These methods are as follows:

2.15.2.1 Nucleic acid hybridization assay

Nucleic acid hybridization assay use to detect human papilloma virus DNA include: 1- non-amplified and 2- signal amplified hybridization assay. Southern blotting, dot-blot hybridization and in situ hybridization are types of non-amplified hybridization

assay. While hybrid capture assay is type of signal amplified hybridization (Carvalho Maria Odete O *et al.,* 2003; Zaravinos *et al.,* 2009). One of main advantage of these techniques is they provide high quality data. Drawback of this approach are, it require enormous purified DNA, less sensitive and laborious.(Villa and Denny 2006a; Kelesidis *et al.,* 2011).

2.15.2.2 PCR detection method

Polymerase chain Reaction is very sensitive and specific technique used for amplification of specific DNA. DNA polymerase is the key enzyme that recognizes and extends oligonucleotide primers to generate the exact DNA product of interest. Billions of copies can be amplified from single dsDNA in a polymerase chain reaction (Lorenz 2012; Garibyan and Avashia 2013).

L1 which encodes major viral capsid proteins is primer target site in human papillomavirus genome (Burd 2003). Genotype specific primers, restriction-fragment length polymorphism and direct sequencing techniques are used to identify human papillomavirus genotypes (Doorn *et al.,* 2001). PCR is a sensitive technique even a tiny contamination in sample may lead to false interpretation of results (Smith Cindy J and Osborn 2009).

2.15.2.3 mRNA detection method

Messenger RNA (mRNA)HPV significantly shows high sensitivity and specify for accurate assessment of precancerous lesion, cervical intraepithelial neoplasia and any cervical malignancy (Macedo *et al.,* 2019). Many studies have reported mRNA detection method for HPV oncogenic proteins expression (Verdoodt *et al.,* 2013).

mRNA expression of HPV oncogene E6 and E7 use to validate active viral infection where malignancy occur (Fontecha *et al.,* 2017). Techniques used to detect HPV mRNA are reverse transcriptase polymerase chain reaction (RT-PCR), transcription based amplification system (TAS), Nucleic acid sequence based detection (NASBA) (Haedicke and Iftner 2016). E6 and E7 mRNA can be detected in high risk human papillomavirus types by PreTect HPV proofer assay. (Rijkaart *et al.,* 2012) NASBA based detection of human papillomavirus exhibits more specificity but is less sensitive (Boulet *et al.,* 2010). The Aptima HPV assay is more sensitive and can

identify fourteen high risk HPV 16,18,31,35,39,51,52,56,58,59,66, and 68 respectively (Ratnam *et al.,* 2011; Origoni *et al.,* 2015)**.**

2.15.2.4 DNA sequencing

Development of sequencing technology has significant impact on analyzing genomic sequence of various organisms (Shendure and Ji 2008). DNA sequencing is a sensitive method used to determine precise sequence of nucleotide base in DNA. This technique is used in many biological, medical and forensic sciences (Tipu and Shabbir 2015).

First technique for sequencing originated around 50 years back, since than the sequence technology has been continuously evolving and on basis of upgrading divided into First generation sequencing method, second generation and third generation sequencing method (Heather and Chain 2016; Kchouk *et al.,* 2017). First generation sequencing technique involves Maxam-Gilbert method and Sanger (dideoxy) method. Sanger sequencing is earliest technique used to describe sequencing of genome (Sanger 1992). High quality can be obtained by Fluorescent labeling of nucleotides incorporated into Sanger sequencing.(Smith Lloyd M *et al.,* 1986; Lee Linda G *et al.,* 1992). Primary advantage of next generation sequencing is increase sensitivity to identify from less frequency variants (Stasik *et al.,* 2018).

Epidemiological studies reveal that human papillomavirus genotyping is very essential for accurate diagnosis and for improving vaccine (Ermel *et al.,* 2010). Clinically, human papilloma virus identification is based on PCR which involves DNA detection kits. These kits may be unable to detect any multiple infection or rare or new HPV types. (Eklund *et al.,* 2010; Mori *et al.,* 2011). Next generation method is high-throughput approach for exact HPV genotyping.(Arroyo *et al.,* 2013).

2.16 Prevention

2.16.1 Primary prevention

Primary preventions to prevent HPV infection involve some changes in lifestyles. These primary prevention strategies can reduce the risk of HPV infection. Delay in early age sexual intercourse, allowing transformation zone maturation so it is less susceptible to HPV infection (Plummer *et al.,* 2012). Risk of cervical cancer

increases with multiple sexual partners, in order to avoid infection it is important to limit number of sex partners (Ranjeva *et al.,* 2017). Another prevention strategy is use of condoms in each sexual activity as it l the chances of transmitting HPV infection (genital warts and cervical cancer) (Lam *et al.,* 2014). Circumcision of neonates and adult may provide protection against sexually transmitted infection (Morris and Hankins 2017). Limit use of tobacco as Smoking is associated to increase risk of cervical cancer, (Xi *et al.,* 2009; Kariche *et al.,* 2018).

2.16.2 Secondary prevention

Secondary preventions to avoid HPV infections involve regular cervical screening and physical examination. Pap screening can identify any cytological alternation in cervical epithelium lining and it is useful to detect HPV infection and stage of disease which aids in treatment. Clinical and Physical examination of female patients by health care provider is also important to detect any abnormality in lower genital area. Female patient must be encourage to so self-examination and report any abnormal finding to their gynecologist (Diaz 2008).

2.17 Vaccines

Last two decades are very important in terms of battle against cancer, after recognizing association of human papilloma virus with cancer. The development of vaccine to prevent HPV infection is an historical milestone in medical field. This prophylactic vaccine is powerful tool to prevent diseases associated with human papilloma virus (Bősze 2013).

Currently two prophylactic human papillomavirus vaccines are licensed, these are Bivalent and quadrivalent vaccine. These vaccines comprised of virus like particles (VLPs). In developed countries these vaccines are given to naive girls in routine vaccination schedule. Gardasil is bivalent vaccine incorporates for type HPV 16 an HPV 18 and cervarix is quadrivalent vaccine incorporates four HPV type i.e. HPV 6,11,16 and 18. (Erickson et al., 2014; Harper and DeMars 2017). These vaccines are injected intramuscularly. It is observed that bivalent vaccine provide protection for 6.4 years and 8.5 years in case of quadrivalent vaccine. There are ongoing clinical trials for nine valent vaccines and it is believed that upcoming vaccine may cover

more HPV genotypes to prevent up to 80% of carcinoma of cervix (David et al., 2009; Frazer et al., 2011).

2.18 Treatment

Cervical cancer is a preventable disease. Screening programs have reduced the incidence and mortality rate of cervical cancer in developed countries but mortality rate is much higher in developing countries (Oaknin *et al.,* 2012). The treatment of cervical cancer patient is based on the diagnosis provided by health care provider (gynecologist, oncologist, surgeon and radiotherapist). Treatment of cervical cancer may involves surgery of effected site, radiotherapy and chemotherapy or they can be used in combination (Kokka *et al.,* 2015; Bhatla *et al.,* 2018). Radiation therapy was standard treatment to cure early stage or FIGO stage IV cervical cancer till 1990 (Rose *et al.,* 1999).

The treatment of cervical intraepithelial neoplasia comprised of ablative or excisional techniques. Ablative technique is more feasible than surgical excision techniques in poor resource settings. Ablative procedure involve in situ destruction of infected tissue, hence there is no tissue specimen for histopathological diagnosis. However, excisional procedure involves removal of part or whole cervix. The excised part can be evaluated by histopathologists for definite diagnosis (Castle *et al.,* 2017; Hoffman *et al.,* 2017).

Ablative procedure involve cryotherapy ,carbon dioxide laser ablation and use of chemo-destructive agents (tri-chloroacetic acid and bi-chloroacetic avid) (Diaz 2008). Previously, hysterectomy was usually done for cervical intraepithelial lesions, cervical colposcopy should be performed before hysterectomy (Keys *et al.,* 1999; Schockaert *et al.,* 2008). The surgical exclusion of cancerous lesion from transformation zone is done by Cold-knife conization (CKC) aids in histo-pathological analysis of tissue. Tissue specimen can also be obtain by Large loop excision of transformation zone (LLETZ) procedure (Melnikow *et al.,* 2009; Santesso *et al.,* 2016).

3. Materials and Methods

The experimental work was divided into following phases:

3.1 Phase 1: Evaluation of socio-demographic characteristics

It was a descriptive cross-sectional study. In this phase sociodemographic data was collected from female patients to assess the epidemiological factors relating to the etiology of the disease (low socioeconomic class, early marriages, multiparty and poor standard of hygiene etc.). Such information was helpful in structuring powerful preventive public health strategies to decrease carcinoma and related mortality.

3.1.1 Study Population

The study population consisted of 250 women of age group 21–60 years old attending Outpatient Department of Gynae/Obstetrics in Pakistan Institute of Medical Sciences (PIMS), Islamabad and Armed Forces Institute of Pathology, Rawalpindi (AFIP), since January 2013 till December 2014.

3.1.2 Ethical approval

The study has been approved by ethical committee of Quaid-i-Azam University, Islamabad and National Institute of Health, Islamabad.

3.1.3 Demographic data collection

A questionnaire was made to record baseline information and potential risk factors, based on published literature on cervical cancer screening (Figure 3.1). Socio-demographic data comprised of social status (name of female patient, age, ethnicity, marital status, education, and employment status), medical history, clinical gynecological complaint and risk factors for human papilloma virus infection. This particular data was used for possible risk assessment. It also includes socio-economic information of patient husband. A written consent was took from female patients before the procedure. Females being Pakistani, married complaining abnormal vaginal discharge and suspicious looking cervix are included in study and females with pregnancy or menstruation were excluded. Patients were also informed about the importance of screening tests and its benefits and associated risk factors.

Socio-demographic performa

Female patient				Patient's husband			
Name				Name			
Age				Age			
Area	Rural	Urban		Area	Rural	Urban	
Ethnicity				Ethnicity			
Marital status	Married	Divorced	Widow	Marital status			
Age at marriage				Age at marriage			
Number of marriages				Number of marriages			
Age at 1st pregnancy							
Duration of marriage				Duration of marriage			
Parity				Parity			
Occupation	Employed	Unemployed		Occupation	Employed	Unemployed	
Education	Illiterate	Primary	Secondary	Education			
	Higher secondary	Graduate	Post graduate				
Socioeconomic status	Low	Middle	High	Socioeconomic status	Low	Middle	High
History of STD				History of STD			
Smoking	Yes	Never	Stopped	Smoking	Yes	Never	Stopped
Contraception	Yes	No		Contraception	Yes	N0	
Any information about cervical cancer screening test							
Gynecological complaint							
Contact information							

Figure 3.1: Proforma used for recording socio-demographic details

3.1.4 Data analysis

Data was analyzed by using IBM SPSS statistics 21. Descriptive statistics were applied for data analysis. Means and standard deviations were calculated for age and frequencies were calculated for all qualitative variables in present study.

3.2 Phase 2: Pelvic examination and Screening test

Pelvic examination and screening test was performed after sociodemographic data collection from female patients. This study was conducted in Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan Institute of Medical Sciences (PIMS) and Department of Histopathology in National Institute of Health (NIH), Islamabad, since January 2013 till December 2014.

3.2.1 Inclusion and exclusion criteria

250 sexually active females, 21–60 years old who had attended Outpatient Department of Gynae/Obstetrics in Pakistan Institute of Medical Sciences (PIMS), Islamabad and Armed Forces Institute of Pathology, Rawalpindi (AFIP), since January 2013 till December 2014were recruited to study only 118 cases were selected for further investigations on basis of exclusion and inclusion criteria.

Inclusion criteria involved being Pakistani, married women, abnormal vaginal discharge, clinically suspicious looking cervix and no previous gynecological malignancy diagnosis. Exclusion criteria involved pregnancy, ongoing menstruation, and history of cervical abnormalities, amputated cervix, and previous total hysterectomy.

3.2.2 Pelvic examination and sample collection

Pelvic examination was done for 250 female patients only 118 cases were selected for further investigations on basis of exclusion and inclusion criteria. A written consent was obtained from patients before doing the examination. Pelvic examination consists of following steps;

3.2.2.1 Examination of vulva and vagina

First step involved examination of vulva for any graze, abnormalities, infection wart and it was documented. A direct visual inspection of the vulva, vagina and cervix was

performed to observe any visible abrasion by inserting Cusco's bivalve vaginal speculum in the lithotomy position. Any mucus or vaginal discharge was removed by using cotton gauzes.

3.2.2.2 Cervical scrapings

Second step involved examination of cervix, the surface of cervix was cleared with help of cotton gauze or swab to remove any discharge. Proper visualization of the transformation zone, squamous columnar junction and the external OS was assured before taking the sample. Pap smear was obtained by gentle scrapping of the squamocolumner junction throughout the circumference by the help of Ayre's spatula. Spatula was rotated in 360° to ensure adequacy of sample. The cervical material on spatula was immediately transferred on a glass slide and a smear was prepared, fixed in 95% alcohol immediately and later it was stained by Papanicolaou stain method.

Papanicolaou stain method involved following steps; fixation in 95% ethanol for 15 minutes followed by 70 % ethanol for 2 min and 50% ethanol for 2 min respectively. Later it was rinsed with tap water and hematoxylin was added and allowed to stand for 3 to 4 minutes. Hematoxylin was removed by rinsing it with tap water briefly. Dipped in 70% ethanol for 1 min and 50% ethanol for 1 min and later washed with 95% ethanol. Following step dehydration to 95% ethanol it was stained with orange G stain for 10 seconds and subsequently washed with 95% ethanol. Later it was stained with Eosin Azure stain for 2 minutes. The sample was rinsed with absolute ethanol and finally with xylene. It was dipped in xylene till it was cleared and mounted in DPX.

3.2.2.3 Visual inspection with acetic acid

After taking Pap smear final step in pelvic examination involved visual inspection with acetic acid (VIA). In this method 5 % freshly prepared acetic acid solution was applied on cervix with help of cotton swab. A positive VIA result was interpreted if a well-defined opaque aceto-white mark appeared on squamocolumner junction.

3.2.3 Reporting of Pap smears

The Bethesda system was used for reporting Pap smear test. It has four main categories of reporting smear; atypical squamous cell of undetermined significance

(ASCUS), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), squamous cell carcinoma (SCC). Slides with dysplasia (mild, moderate, severe), carcinoma in situ (CIS) or squamous cell carcinoma were reported positive histopathologically.

3.3 Phase 3: HPV Screening and genotyping

Human papillomavirus screening mainly focused on detection of high grade precursor lesions and genotyping involved recognition of high risk HPV types on basis of their prevalence in cervix cancer. This phase involved detection and genotyping of high risk HPV types causing cervical cancer in Pakistani women.

3.3.1 Study conducted

Present study was conducted in Virology Department of National Institute of Health (NIH), Pakistan and approved by the Ethical Review Committee of NIH. Patients provided informed written and agreed with their data being included in the present study.

3.3.2 Sample collection

A total of 105 formalin-fixed paraffin embedded (FFPE) cervical carcinoma biopsy samples collected between 21-80 years old women from January 2013 to December 2015 were used for the assessment of human papilloma virus infection. These FFPE biopsy specimens were pathologically confirmed by histopathologists as chronic cervicitis, adenocarcinoma, and squamous cell carcinoma of cervix. These samples were obtained from two major government hospitals including Pakistan Institute of Medical Sciences, Islamabad and Armed forces Institute of Pathology, Rawalpindi. These hospitals are visited by patients that have different social background and ethnicities in different regions of Pakistan (Khyber Pakhtunkhwa, Punjab, Gilgit Baltistan and Azad Jammu and Kashmir).

Before sectioning, FFPE blocks were placed for approximately 30 minutes in -20°C. Tissue biopsy block samples were cut into 15-20 µm cross sections on microtome (CUT6062,SLEE Mainz) and placed in to 1.5mL Eppendorf tube. For each specimen 12- 15 sections were taken and only 4-5 sections were subjected for assessment of HPV infection. Blades of microtome were properly cleaned with 70% ethanol to

avoid cross contamination between each specimen. Samples were carefully transported and stored in lab under 4ºC temperature.

3.3.3 Extraction of Genomic DNA and Detection of HPV Infection

The HPV DNA was extracted using the commercial kit known as from Total all nucleic acid isolation kit for FFPE (Am1975 Ambion, Invitrogen). Before conducting the actual extraction, the samples underwent xylene-dewaxing. The sections were deparaffinized in 1 mL of xylene and shaken vigorously for 2 min followed by centrifugation at 12,200× g for 10 min.

The supernatant was removed and the pellets were then washed again in xylene to avoid carryover of residual paraffin. The pelleted tissue was treated with 1 mL of 100% (w/v) ethanol followed by centrifugation (16,200× g for 10 min) so as to remove residual xylene from the tissue. The supernatant was discarded and the tissue washed with 1 mL of 70% (w/v) ethanol before re-centrifugation at 16,200× g for 10 min. The extraction was performed following the protocol provided by the manufacturer using micro-columns equipped with collecting tubes. Then extensive Protease digestion was carried out and DNA was recovered. This DNA was purified, eluted and stored at -20°C until further amplification.

3.3.4 Polymerase chain reaction for amplification of β globin gene

DNA was amplified by PCR and its quality was evaluated by amplification of housekeeping β globin gene using specific primers (Table 1). β globin PCR was conducted in a 25 µL PCR reaction mixture contained 10 µL of extracted DNA, 1.5 µL of 25mM $MgCl_2$, 2.5 µL of *10X Taq Buffer* (100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 µL of 2mM dNTPS, 10µM of each oligonucleotide primers (β globin Forward and Reverse Primers) 0.5 U *Taq DNA polymerase* (Thermo Fisher scientific, Carlsbad, CA, USA). The PCR thermal profile was 95ºC for 5 min, and 40 cycles, 94ºC for 45 sec, 55 ºC for 45sec, 72ºC for 45 sec, and final extension at 72ºC for 5 min.

HPV screening of all β globin gene positive samples was carried out using nested Polymerase Chain Reaction (PCR) with My09/My11 and GP5+/GP6+ primer sets (target a conserved sequence in HPV L1gene) for FFPE tissue samples. The

sequences of primers used in each reaction mix with predicted amplicon size were shown in Table 3.1.

3.3.4.1 Nested Polymerase chain reaction

The first round PCR was done using MY09/MY11 primers in total 25 µL reaction mixture containing 10 µL of extracted DNA, 4 µL of 25mM $MgCl_2$, 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 µL of *10X Taq Buffer* (100 mM Tris-HCl (pH 8.8 at 25°C), 2.5 µL of 2mM dNTPS, 10µM of each oligonucleotide primers, 0.5 U *Taq DNA polymerase* (Thermo Fisher scientific, Carlsbad, CA,USA).The PCR thermal profile for 1st round PCR was 94ºC for 3 min, and 40 cycles 94ºC for 1 min , 55ºC for 2 min , 72ºC for 2min, and final extension at 72ºC for 10 min.

The second round PCR was done using GP5+/GP6+ primer sets. A total of 25 µL reaction mixture contained 2 µL of 1st round PCR product, 1.5 µL of 25 mM $MgCl_2$, 2.5 µL of *10X Taq Buffer* (100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 µL of 2 mM dNTPS, 10µM of each oligonucleotide primers , 0.5 U *Taq DNA polymerase* (Thermo Fisher scientific, Carlsbad, CA, USA).The PCR thermal profile was 95ºC for 5 min, and 40 cycles, 94ºC for 45 sec , 42ºC for 45sec, 72ºC for 45 sec , and final extension at 72ºC for 5 min.

The amplified PCR products were separated by electrophoresis on 1.5% agarose gel stained by ethidium bromide. Amplicon sizes were estimated by comparison with 100 bp molecular marker (Fermentas gene Ruler 100bp plus DNA; Ladder Catalog number: SM0322) and were visualized on UV trans-illuminator. (Bio Rad).

Table 3.1: List of primers.

Primer Name	Primer Sequence (5'-3')	Target	Amplicon Size (bp)
β-globin	5'-GAA GAG CCA AGG ACA GGT AC-3'	β-Globin	268
β-globin	5'-CAA CTT CAT CCA CGT TAC ACC-3'	β-Globin	268
MY09	5'-CGT CCM ARR GGA WAC TGA TC-3'	L1	450
MY 11	5'-GCM CAG GGW CAT AAY AAT GC-3'	L1	450
GP5+	5'-TTT GTT ACT GTG GTAGATACTAC- 3'	L1	150
GP6+	5'-GAAAAATAAACTGTAAATCATATTC-3'	L1	150
HPV16 F	5'-CTG CTT TTA TAC TAA CCG G-3'	L1	217
HPV16 R	5'-AAG GCC AAC TAA ATG TCA C-3'	L1	217
HPV18 F	5'-CCT TGG ACG TAA ATT TTT GG-3	L1	115
HPV18 R	5'-CAC GCA CAC GCT TGG CAG GT-3'	L1	115

3.3.4.2 Genotyping PCR for High risk HPV 16 and18

DNA samples found positive for GP5+/GP6+ were further analyzed to identify type 16 and type 18 HPV genotypes using specific HPV 16 and18 primer sets (Table 3.1). This PCR was carried out in 25µl PCR reaction mixture containing 10µl of extracted DNA, 2.5 µL of 2 mM deoxy nucleotide triphosphates (dNTPs), 2.5 µL of 10X *Taq* Buffer (100 mM Tris-HCl (pH 8.8 at 25 °C), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 µL of each primer (HPV16 or HPV 18 Primer sets), 0.5 U of Taq DNA polymerase (Thermo Fisher scientific, Carlsbad, CA, USA). PCR thermal profile was 95 ºC for 9 min followed by 35 amplification cycles with a 30 seconds denaturation at 95 ºC , annealing for 30 seconds at 55 ºC (for HPV16), and 58 ºC (for HPV18), and extension at 72 ºC for 30 seconds. A final extension at 72 ºC for 7 minutes was performed. The amplified products for HPV 16/18 were visualized on 2% agarose gel stained with ethidium bromide.

3.4 Phase 4: DNA sequencing

Detection of HPV with help of PCR allows the determination of HPV infections and high risk types HPV 16 and 18 that is useful for better diagnosis and treatment plans for patients. This phase involved nucleotide sequencing of HPV untypeable isolates because it may possibly that some new emerging genotypes or variants are circulating in the community that are needed to be explored.

3.4.1 Polymerase chain reaction for HPV

HPV positive DNA samples for GP5+/GP6+ primer set were further subjected to DNA sequencing for human papillomavirus genotypic characterization. PCR protocol with thermocyclic parameters were shown in table 3.1. The amplified product electrophoresed on 2% agarose gel along with a 100-bp DNA ladder and results were interpreted.

3.4.2 Purification of amplified products

The amplified products synthesized as a result of nested PCR was purified by using the AxyPrep^{TM} Mag PCR clean-up. The AxyPrep^{TM} Mag PCR clean-up kit used special paramagnetic bead technology for quick and high-throughput refinement of PCR products. The method involved binding, washing and elution stages and removed salts, enzymes, primers and nucleotide from amplified PCR product. It comprised of following steps:

1. **Preparation step:** A 300µL round bottom plate was utilized because the volume of PCR reaction exceeded from the size of normal PCR plate. The bottle of AxyPrep^{TM} Mag PCR clean-up was shaken gently before use to re-suspend magnetic particles.
2. **Binding step:** 90µL of reagent was added in PCR reaction (the calculation was based on formula 1.8X volume of PCR reaction mixture). It was mixed thoroughly with help of pipette and incubated at room temperature for 5 minutes. The reaction plate was placed on Magnet plate and solution was cleared in 3 minutes. Clear solution was aspirated without disturbing the beads.

3. **Washing step:** Washing step involved addition of 200 µL of 70 % ethanol followed by incubation for 30 seconds and aspirated. Washing step was repeated two times.
4. **Elution step:** In elution step, reaction plate was removed from Magnetic plate and 40µL of elution reagent was added and Mixed well with pipette, the plate was again placed for 1 minute on Magnetic palate to spate beads from solution mix and supernatant was transferred to new tube and stored at -20 ^{0}C until further analysis.

3.4.3 Cycle sequencing/Nucleotide sequencing PCR

The purified PCR products were subjected to cycle sequencing PCR. A total of 20 µL reaction was carried out that contained 1 µL of the purified PCR product, 1 µL of 5 µmolar sequencing primer (GP6+), 1µL Big Dye Terminator cycle sequencing kit (version 3.1; Perkin Elmer Applied Biosystem) 3.5 µL 5× buffer, and 13.5 µL water. The thermal profile was 95˚C for 25 sec, and 30 cycles of amplification, 95˚C for 15 sec, 50˚C for 20 sec and 60˚C for 1 min.

3.4.4 Purification of DNA sequencing samples

The AxyPrep^{TM} Mag dye clean up kit was used for dye terminator clean up into the sequencing reactions. It was important step before sequencing to avoid any carryover of dye which can affect the results. It involves following steps:

1. **Preparation step:** Paramagnetic beads in solution were re-suspended by brief vortexing before using.
2. **Binding step:** It involved addition of 40 µL of reagent to each sample followed by addition of 85% ethanol. The sample plate was placed on Magnetic plate for 5 minutes or so, until solution clears. On sides of magnetic plate a crescent was formed and supernatant was aspirated.
3. **Washing step:** In each well 100µL of 85% ethanol was dispensed and incubated for 30 seconds. Aspirate ethanol without touching beads.
4. **Elution step:** Plate was removed from magnet and 40µL of reagent was added and incubated for 5 minutes. Beads were separated from elution buffer by placing sample plate back on magnet. 35µL of clear supernatant was transferred to new plate for further analysis.

3.4.5 Sequence analysis

3.4.5.1 Editing and Asembly of sequence

The reaction mixture was subjected on an automated ABI 3130 Genetic Analyzer. Sequence data was generated and sequences were assembled and edited by using Sequencher 4.9 software (GeneCodes) (http://www.genecodes.com).

3.4.5.2 Basic Local Alignment Search Tool for online Sequence Data collection

The identification of obtained sequences was verified by using online BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

3.4.5.3 Construction of Phylogenetic tree

Phylogenetic analyses of the HPV L1 gene sequence were performed to investigate the possible genetic relationships and diversity among study isolates and sequences of other HPV types. Phylogenetic tree was constructed by aligning study HPV strains with nucleotide sequence of their closely relative strain retrieved from GenBank. The prototypes sequence were downloaded from the Papilloma Virus Episteme (PaVE) database (http://pave.niaid.nih.gov)

Sequence alignments were prepared by using ClustalW. The phylogenetic tree was constructed using the maximum likelihood algorithm method and Kimura 2-parameter model with 1000 bootstrap replicates using Molecular Evolutionary Genetics Analysis version.

3.5 Phase 5: Keratinocyte and fibroblast monolayer cell culture.

The wound healing response facilitated establishment of HPV genomes. The phases of wound healing in the context of human papillomavirus infection can be studied *in vitro* using keratinocytes cultured in monolayer. This phase involved establishment of J2-3T3, Normal Immortalized Human Keratinocytes (NIKS) and NIKS HPV 16 cell lines Present study was conducted at Department of Pathology, University of Cambridge, United Kingdom since February 2015 till July 2015.

3.5.1 Buffer and reagents

All chemicals used in this phase were purchased from Fisher Scientific (UK), Sigma-Aldrich (UK) and BDH supplies (UK) and buffers were acquired from NIMR media facility. Buffer and reagents used in the study are shown in table below:

Table 3.2: Reagents and Buffers

Sr. No.	Name of Reagent and Buffers	Components
1	1x Phosphate buffer saline (PBS)	0.025% KH_2PO_4 , 0.025% KCl, 1% NaCl and 0.14% Na2HPO.
2	50x Tris acetate EDTA	57.1 mL glacial acetic acid, 242g Tris base and 18.6 g Ethylenediaminetetraacetic acid (EDTA)
3	Trypsin-versene	0.01% EDTA, 0.8% NaCl, 0.12% Na_2HO_4, 0.02% KCl, 0.13% trypsin, 0.001% phenol red and 0.02% KH_2PO_4
4	Penicillin/ streptomycin	1% (V/V) streptomycin and 0.6% (V/V) penicillin

3.5.2 Basic monolayer cell culture method

3.5.2.1 Cell lines

1- J2-3T3 is an immortalized fibroblast cell line and was used as feeder layer for supporting the growth of normal immortalized human keratinocyte cell lines.

2- NIKS is a cutaneous foreskin keratinocyte cell line and were used as host cells. It was used as negative control in NIKS HPV 16 experiments.

1. **NIKS HPV16 cells**

HPV 16 harboring NIKS cell lines were formed through twice independent transfections performed by Kenneth Raj and Qian Wang, NIMR London. Normal Immortalized Human Keratinocytes were co-transfected with HPV16 wild type genome and a vector pcDNA6 having resistance gene Blasticidin. After several days of Blasticidin selection (6µg/mL) HPV16 clonal cell lines were obtained. Cells were cultured in 6-well plate once individual colonies were visible and expanded to create a cell stock.

3.5.2.2 Supplements and media for cell Culture

Media used for cell line culturing and freezing of cells for long period is shown in table 3.3. Supplements for media were also prepared, followed by filter sterilization using membrane filter unit (0.2µm). Supplements were than aliquoted (5mL) and stored at -20ºC for further use.

Table 3.3: Media and Supplements.

Sr. No.	Cell line type	Media	Components of Media
1	J2-3T3	Dulbecco's Modified Eagle Medium (DMEM)	500 mL high glucose DMEM (sigma-Aldrich; D6429). 1% (v/v) streptomycin/penicillin and 10% (v/v) Fetal bovine serum.
2	NIKS and NIKS HPV16 clones	F-medium Incomplete (FI)	500 mL F-Medium (1 portion of high glucose DMEM and 3 portions of F12-Hams) (PAA; T15-355), 24 µg/mL Adenine (Sigma-Aldrich; A2786), 0.4 µg/mL hydrocortisone (Calbiochem; CAS 50-23-7), 1% (v/v) strep/pen, 5% (v/v) Fetal Bovine Serum (Biosera; S1900-500), 5 µg/mL Insulin (Sigma-Aldrich; 14011) and 8.4 ng/ml cholera toxin
3	NIKS and NIKS HPV 16 clones	F-medium complete (FC)	500 mL FI medium and 10ng/mL epidermal growth factor (EGF) (R&D Systemms;236-EG)
4	J2-3T3	J2-3T3 Freezing medium	5% (v/v) Dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2650) and 95% (v/v fetal bovine serum
5	NIKS and NIKS HPV 16 clones	NIKS Freezing medium	10 % (v/v) Dimethyl sulfoxide (DMSO) (Sigma-Aldrich; D2650) and 90% (v/v) fetal bovine serum

3.5.2.3 Maintenance of monolayer cell

1. J2-3T3

J2-3T3 feeder cells were cultured in 75 cm flasks (Corning; UK) having 10mL of Dulbecco's Modified Eagle Medium (DMEM) at 37ºC in 5% carbon dioxide (CO_2). Cells were allowed to grow up to 80% confluency and then splited to (1:20) to a new 75cm flask.

To Harvest J2- 3T3, the fibroblasts were first washed with PBS (10 mL) and then 1mL of trypsin versene was added. Cells along with trypsin versene were incubated for 2 minute at 37ºC and 9 mL of 3T3 media was dispensed. Later, fibroblast cell suspension was transferred to another flask for culturing. Fresh media was given to cell on every 3-4 day.

In order to store J2-3T3 for long period, cells were spun down at 1400 rpm for 5 minutes in MSE Mistral 1000 centrifuge and re-suspended in J2-3T3 freezing media (2 mL) and transferred to cryovial and stored at -80ºC till further use. J2-3T3 cell cultures passaged more than 20 times must not be used in further experiments.

2. NIKS and NIKS HPV 16 cell lines

Prior to NIKS cell culturing, J2-3T3 cells were irradiated (60Gy). A minimum of $1.4x10^6$ irradiated cells were seeded in 75 cm flasks containing 10 mL of FI media and left to attach for at least two hours at 37ºC. NIKS and NIKS HPV 16 cell lines were cultured on these gamma-irradiated J2-3T3 cells. Approximately $4x10^6$ NIKS /NIKS HPV16 were seeded in flask containing 10 mL of FI medium. FI media was replaced by 10 mL FC medium after 24 hours and is changed every other day.

Keratinocytes were harvested once they were 80% confluent. The FC media was aspirated followed by addition of 10 mL of PBS to wash the cells. PBS was removed and 2 mL of trypsin versene was dispensed in flask and incubated for 1 min at 37ºC. The flask was gently tapped many times to detach feeder layer from keratinocytes and later aspirated. The keratinocytes were incubated at 37^0C with 2 mL of trypsin versene for 10 minutes till cells were dislodged. FI medium (8mL) was added in flask having trypsin and a counted suspension of cells were transferred into new flask having gamma-irradiated feeder cell layer. For long term storage, keratinocytes were spun down 1400 rpm for 4-5 minutes and in 1mL of freezing medium $2x10^6$ cells were re-suspended and shifted to cryovial at store at -80ºC.

3.5.2.4 Cell Count of NIKS and NIKS HPV 16 cell lines.

After harvesting, NIKS, NIKS HPV16 cells were counted by using a Beckman Z1 Coulter counter (Beckman Coulter, UK). The procedure involved addition of 1mL of cell suspension in 9 mL of isotone solution in a cuvette (VWR International Ltd; 720-0812). The program for coulter counter was optimized for NIKS specifically (Erin Issacson NIMR, London, UK). It can count NIKS cells size between 11- 20 µm, however, for cells larger than 11 µm the coulter counter parameters were adjusted to count all cells above size 11µm.

3.5.2.5 Extraction of total genomic DNA from NIKS HPV16 cell lines

Before DNA extraction, cells were washed with 10 mL of PBS and spun down at 3000 rpm for 5 minutes in micro centrifuge. Later 200 µL of PBS was added and transferred in to 1.5 mL Eppendorf tubes and stored at -20ºC for immediate use or -80ºC for long term use. The extraction of total genomic DNA from NIKS/NIKS HPV16 cell lines was done by using the QIAamp DNA Blood Mini Kit (Qiagen, UK). It involved following steps;

1. In 200 µL of sample, 20 µL of proteinase K, 4 µL of RNAase.
2. 200 µL Buffer AL was added and mixed by pulse vortexing for 15 seconds and incubated at 56ºC for 10 minutes.
3. The 1.5mL tube was briefly centrifuged to remove any droplet from inside of lid.
4. 200 µL of 96-100% ethanol was added followed by pulse vortexing for 15 seconds and briefly centrifuged.
5. The mixture was carefully applied to QIAamp Mini spin column and centrifuged at 8000rpm for 1minute and filtrate was discarded.
6. Later, Buffer AW1 (500 µL) was added and spun down at 8000rpm for 1 minute. Filtrate was discarded and column was placed into a new collecting tube (2 mL).
7. 500 µL of Buffer A1 was added at this stage and centrifuged at full speed (14000rpm) for 3 minutes.

8. The spin column was placed in new 1.5 mL micro centrifuge tube and 50 µL of Buffer AE was added and incubated for 1 minute at room temperature and later centrifuged for 1 minute at 8000 rpm.
9. Extracted DNA was stored at -20ºC till further use.

3.5.3 Quantification of genomic DNA

Nano drop 8000 spectrophotometer was used for DNA quantification.

3.5.3.1 Quantitative Real-time PCR (qPCR)

HPV 16 copy number per cell was determined by using quantitative real-time polymerase chain reaction (qPCR). GAPDH a house keeping gene was used as an internal control.

3.5.3.2 qPCR primers, Reagent cocktail and cycle parameters

GAPDH primers were designed in such a way that it contained minimal amount of homology to the sequence of mouse GAPDH DNA. This was to guarantee that any impurity by mouse J2-3T3 fibroblasts should not reflect in readouts. Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) was used to using an ABI-7500 Real Time PCR. Sequence of forward and reverse primers is shown in table3.4.

Table 3.4: Primers used in real time PCR.

Primer Name	Primer sequence 5'-3'
GAPDH F	5'-CCTCCCGCTTCGCTCTCT-3'
GAPDH R	5'-CTGGCGACGCAAAAGAAGA-3'
HPV 16 F	5'-GACTATCCAGCGACCAAGATCAG-3'
HPV 16 R	5'-CTGAGTCTCTGTGCAACAACTTAGTG-3'

Table 3.5: Real time PCR cocktail.

RT-PCR cocktail	GAPDH (µL)	HPV 16(µL)
Forward primer	1.75µL	1.75µL
Reverse primer	1.75µL	1.75µL
Syber green	1.75µL	1.75µL
Molecular water	8µL	8µL
Total volume	13.25µL	13.25µL

Table 3.6: qPCR parameters

qPCR cycle parameter			
Step	**Number of cycles**	**Time per cycle**	**Temperature (°C)**
1	1	2 minutes	50
2	1	15 minutes	95
3	40	15 seconds	95
4	1	1 minute	60
Dissociation parameters			
Step	**Number of cycles**	**Time per cycle**	**Temperature (°C)**
1	1	15 seconds	95
2	1	20 seconds	60
3	1	95 seconds	95

3.5.3.3 qPCR standard curve

A standard curve was generated for every primer set in selected qPCR parameters (Table 3.6), so as to determine the effectiveness and sensitivity of primers. A pDrive that have 300bp of GAPDH open reading frame was utilized as template to create standard curve of GAPDH. To set up a linear relationship, a series of six 10 fold dilutions were plated in triplicate. DNA concentrations were converted into copy numbers to generate a standard equation that would decide all number of GAPDH copies in a reaction.

To generate HPV16 standard curve pTZH16-W12 was utilized as template. In order to build a linear relationship, HPV 16 six 10-fold serial dilutions were plated in triplicate. The concentration of DNA was converted to copy numbers in order to create a standard equation that would determine the absolute number of HPV copies in every reaction. Standard curve was only generated once for GAPDH and HPV 16 as a similar primer stock was utilized for each qPCR. The standard curve equation for GAPDH and HPV 16 was:

Equation of standard curve for GAPDH $y= -3.39x + 40.61$

Equation of standard curve for HPV, $y= -3.33x+ 35.78$

3.5.3.4 Standard curve equation to calculate copy number

There were two significant parameters in GAPDH and HPV16 standard curve equation (y=mx+b). In equation "y" determined the sensitivity of primer while "m" referred to the primer efficiency. A slope -3.33 demonstrated an ideal linear relationship among copy number and ct (cycle threshold) value. For each set of triplicates, cycle thrush hold (ct) value was averaged in order to calculate all HPV 16 copies per cell. To represent pipetting error the software additionally created a standard deviation for each triplicate. If the value surpassed 0.300 the reaction was repeated. Hence, for the acceptable ct value the standard equation was pursued as follows: If y=mx+b than "b" represented y intercept, "y" showed ct value and "m" indicated the slope, at that point In-1x would determine GAPDH and HPV 16 copy number in each reaction. Copy number of GAPDH was divided by four to generate total number of cells in a reaction. Furthermore, for each cell line, HPV copy number was calculated by dividing total HPV copy number by total number of cells.

3.6 Phase 6: Monolayer growth assay for determining effect of epidermal growth factor (EGF) on copy number and gene expression

Monolayer NIKS and NIKS HPV 16 cell lines were subsequently used to evaluate viral copy number, early oncogene expression and growth potential. Hence growth assay tests were utilized to determine the level of proliferation for monolayer NIKS and NIKS harboring HPV 16. In monolayer growth assay different concentrations of EGF were used and these cells were counted in triplicate subsequently on days 1, 3, 5 and 7.

3.6.1 Seeding of fibroblast and NIKS for growth assay with different EGF concentrations

The first step of growth assay involved seeding of 1x 10^5 gamma-irradiated fibroblasts in to each well of 6 well plates (60 mm) followed by addition of 2mL of FI media. The cells were left to attach for 2 hours at 37°C in 5% $C0_2$.

Later, 1x 10^5 NIKS/NIKS HPV16 clones were seeded on bed of fibroblasts and FI media was added followed by overnight incubation at 37°C. Next day FI media was

replaced with 4 mL of FC media and from this point every other day FC media was added till end of experiment. Three different concentrations of EGF were used to culture HPV 16-NIKS in 6 well plates for seven days. These concentrations of EGF were 10ng/mL, 100ng/mL and 500ng/mL respectively.

3.6.2 Harvesting and counting NIKS and NIKS HPV 16 cell lines

Monolayer NIKS/NIKS HPV 16 cells were harvested on day 1,3,5,7 respectively. On cell harvesting day, each well in 6-well plate was first washed with 2 mL of PBS. After washing 1 mL of trypsin was added in each well and incubated at 37ºC with 5% CO_2 for 2 minutes followed by gentle tapping to detach the fibroblasts. Any abundance of fibroblasts was washed away with PBS. In order to remove keratinocytes, 2mL of trypsin was dispensed to each well and incubated for 15 minutes at 37ºC and 5% CO_2. Once keratinocytes were detached, 2mL of FI media was added in every well to make a single cell suspension. The whole cell suspensions from each well were transfer into individual 15 mL Falcon tubes.

In order to count cells, 0.5 mL cell suspension was added into isotone solution (9.5 mL) and cells were counted using Beckman Z1 Coulter counter. Remaining cells were stored in NIKS freezing media at -80ºC.

3.6.3 Extraction of total RNA

For total RNA extraction, harvested cells were washed with PBS and after that placed on ice. The RNeasy Mini kit (QIAGEN 74104) and QIA shredder unit (QIAGEN; 79654) were used for total RNA extraction according to manufacturer instructions. The RNA was than stored at -80ºC till further used.

3.6.4 Preparation of cDNA

Before making cDNA, DNA free kit (AM 1906) was used to remove the genomic DNA from 2µg of total RNA as indicated by manufacturer. Super Script III First-Strand Synthesis Kit (life technologies; 18080-051) was used to carry reverse transcription. 8 µL of RNA along with 1 µL of 10mm dNTPs and 1 µL of primers oligo (dT) were added in 0.5 mL tube, followed by incubation at 65ºC for 5 minutes and afterwards put on ice for 1 minute. cDNA Synthesis Mix was prepared by adding 4µL of 25 mM $MgCl_2$, 2 µL 10XRT buffer, 2 µL of 0.1 M DTT, 1 µL of RNase Out

(40U/µL) and 1µL of Super Script III RT (200U/µL). 10 µL of cDNA Synthesis Mix was added in primer mix and incubated at 50ºC for 50 minutes. Later reaction was terminated by placing tubes at 85ºC for five minutes. After brief centrifugation 1µL of RNase H was dispensed in each tube and incubated at 37ºC for 20 minutes and later cDNA was stored at -80ºC or it can be utilized for qPCR. An extra tube which serves as negative control was prepared by utilizing 2µL of Nuclease free water rather than enzyme.

Table 3.7: Primer used for RT-PCR

Primer Name	Primer sequences 5'-3'
GAPDH F	5'-TGGATATTGTTGCCATCAATGA-3'
GAPDH R	5'-GATGGCATGGACTGTGGTCATG-3'
HPV 16 F	5'-TCAGGACACAGTGGCTTT-3'
HPV 16 R	5'-ACTGCAATGTTTCAGGAC-3'

3.6.5 Quantitative real time PCR (qPCR)

Primer Select software was used to design primers E7, E6, E6*, E1, E2, E4, E8, and GAPDH and purchased from Sigma Aldrich (UK). Primers and there sequences were shown in a table below 3.8.

Table 3.1: Primers used in qPCR to determine gene expression.

Name of Primer	Sequence of primers (5'-3')
E7F	CATGGAGATACACCTACATTGCAT
E7R	ATTTGCAACCAGAGACAACTGA
E6F	ACGGTTGTTGTATTGCTTCTT
E6R	TCACATACAGCATATGGATTCCCA
E6* F	CTGTTGCTTGCAGTACACACA
E6 *R	TTGTTTGCAGCTCTCTGTGCCAT
E1F	TGCTAACATTGCTGCCTTTG
E1R	TTCACTAACACCCTCTCCCC
E2F	CCCTGCCACACCACTAAGTT
E2R	CGACCCATACCAAAGCCGT
E4F	GGCCAAGTGCTGCCTAATAAT
E4R	GGTCGCTGGATAGTCGTCTG
E8F	TGGCCAAGTGCTGCCTAATA
E8R	CTTGGTCGCTGGATAGTCGT

A reaction mixture was prepared having 1.75µL of forward primers and 1.75 µL of reverse primer and 12.5 µL of Power SYBR Green Master-Mix and 8µL of water. To amplify and detect cDNA, the 25 µL of master mix was dispensed in each well of 96-well PCR plate (Thermo Scientific; TUL 962-011N) and placed in ABI-7500 qPCR. For each primer set a new master mix was prepared. Each sample was run in triplicate. qPCR cycle parameters were shown in table previously it also showed dissociation parameters for dissociation curve which showed any primer dimer which may interfere the results.

3.6.6 Determination of copy number and gene expression

The copy number of each transcript of interest in a sample was calculated by standard curve generated for each primer set. Average was done for the triplicate values of primer set and Ct value. To represent pipetting error the software determined the standard deviation for each set. If standard deviation surpassed 0.5 reaction was repeated. Satisfactory Ct average values were utilized to compute copy number and to so as such the standard curve equation was applied to determine value of "X". The

copy number is inverse log of X for example 10X. At the point, when transcript number had been recognized for every primer set the value of HPV 16 were divided by the GAPDH value to standardize the results. The GAPDH copy number was divided by four, to know the total number of cells in every reaction. In order to get complete HPV copy number calculation the total HPV copies number was divided by total number of cells. Ct values obtained from qPCR were imported to Microsoft excel worksheet. Livak and Schmittgen, 2001 method was used to determine relative gene expression between samples.

4. RESULTS

4.1 Phase 1 Results: Evaluation of socio-demographic characteristics

A total of 250 married Pakistani females with gynecological problems included in the present study during the period January 2013 till December 2014. 132 females who did not fit in to the inclusion criteria were excluded. Total 118 females were considered relevant for the screening study and their sociodemographic information was collected.

4.1.1 Geographical distribution

Most females belong to rural areas 68(57.62%) while 50(42.37%) belong to urban areas. The patients in study were from different regions of state. Among 118 female patients the highest portion of patients were Punjabi 44% (n=52) followed by, Pashtuns 26.3% (n=31), Kashmiri 15.3% (n=18), Brusho 5.9% (n=7) and Saraiki 5.9% (n=7) and 2.5% (n=3) belongs to other ethnicity (Figure 4.1).

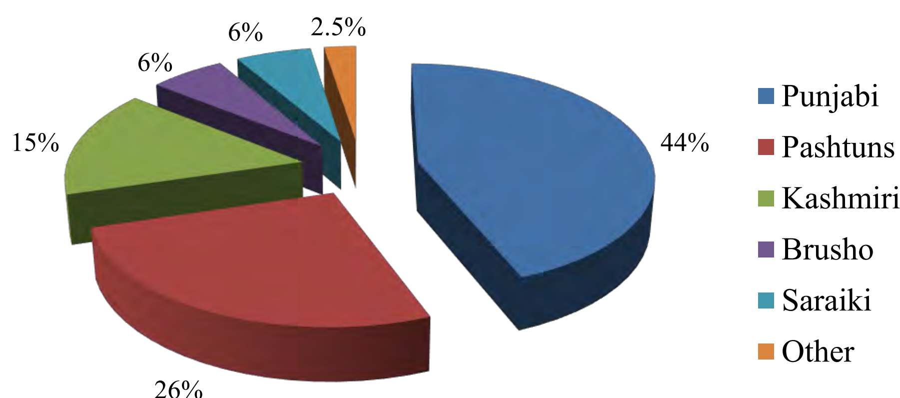


Figure 4.1: Ethnicity wise distributions of female patients.

Sociodemographic data was collected from female patients from 14 districts of Punjab (Barakahu, Chakwal, Dina, Gujar khan, Gujranwala, Islamabad, Jhelum, Jhang, Lahore, Mangla, Rawalpindi, Rawat, Sargodha and Taxila. Most patients were from Rawalpindi 35.5% (n=42/118). Similarly, patients were also from province Khyber

Pakhtunkhwa (Charsda, Hazara, Khanpur, Peshawar and Swabi).Most female patients were from Peshawar (n=29/118). Also, patients with gynecological complaints were also from different districts of Azad Jammu and Kashmir (Bagh, Kotli, Muzaffarabad and Rawalakot) and Gilgit Baltistan.

4.1.2 Age and marital status

The mean age of the female patients in current study was 35.78 ± 9.8 years. Among the 118 patients were 104 married, 11 divorced while 3 widow. Mean age at marriage was 21.77± 4.8 years and mean marital duration was 14.01 ± 10.87 year. Moreover, mean age at first pregnancy was 18.06 ±9.77 years old. Out of 118 females 33 (27.9%) were nulliparous while 85(72%) were parous. Only 40(33.9%) female patients documented use of contraceptives methods. (Table 4.1)

Table 4.1: Social Demographic characteristics of the females who participated in screening study.

Socio-demographic characteristics	Number (%)
Marital status	
Married	104(88.1%)
Divorce	11(9.3%)
Widow	3(2.5%)
Area	
Rural	68(57.62%)
Urban	50(42.37%)
Ethnicity	
Punjabi	52(44.%)
Brusho	7(5.9%)
Sariki	7(5.9%)
Pashtuns	31(26.3%)
Kashmiri	18(15.3%)
Other	3(2.5%)
Socio economic status	
Low	69(58.5%)

Middle	32(27.1%)
High	17(14.4%)
Age at marriage (years)	
≤ 20	69(58.5%)
21-30	35(29.7%)
30-40	14(11.9%)
Duration of marriage(years)	
>5	30(25.4%)
5-10	35(29.7%)
11-20	26(22.0%)
>20	27(22.9%)
Age at first pregnancy (years)	
≤ 20	63(53.4%)
21-30	45(38.1%)
>30	10(8.5%)
Parity	
None	33 (27.9%)
1	20(16.9%)
2or 3	37(31.3%)
More than 3	28(23.7%)
Contraception	
None	78(66.1%)
Yes	40(33.9%)

4.1.3 Education and Employment status

Sixty six (55%) female patients were had education (higher/secondary/ primary) while 52(44%) were illiterate. 35(29.7%) patient's husband had never been to school, 13(11%) had primary, 15(12.7%) secondary education while 16(13.6%) were postgraduate. Detailed education status of husband and wife was shown in Table 2. Majority of females 84(71.1%) included in the study were unemployed while 99(83.8%) of patient's husband were employed. Majority of participants had low income 69(58.5%), only few had high income 17(14.4%) while the rest had middle income 32(27.1%) to live on. Social demographic characteristics of females who participated in the study were detailed in Table 4.2.

4.1.4 Smoking and STD history

Most of the female patients gave history of smoking of husband 78(66%) while 7(5%) had stopped smoking. Among 118 females, 114(96.6%) never smoked. None of them gave history of STI (sexually transmitted infection). Moreover, 115 females had married once as mentioned in table 4.2.

Table 4.2: Knowledge about age, education and employment of the females and their respective husbands.

Age (years)	Female patient (%)	Patient husband (%)
<20	0	12(10.2)
21-30	47(39.8%)	18(15.3)
31-40	41(34.7%)	47(39.8%)
41-50	22(18.6%)	34(28.8%)
>50	8(6.8%)	7(5.9%)
Education		
Illiterate	52(44.1%)	35(29.7%)
Primary	22(18.6)	13(11%)
Secondary	15(12.7)	15(12.7%)
Higher secondary	11(9.3%)	18(15.3%)
Graduate	12(10.2%)	12(17.8%)
Post Graduate	6(5.1%)	16(13.6%)
Employment status		
Unemployed	84(71.1%)	19 (16.1%)
Employed	34 (28.8%)	99 (83.8%)
Smoking		
Yes	4(3.4%)	78(66%)
Never	114(96.6%)	33(28%)
Stopped	0	7(5%)
No. of marriages		
1	115	91(77.11%)
>1	3	27(22.88%)
History of STI	No	No

4.2 Results of Phase 2: Pelvic examination and Screening test

Vaginal discharge was the most frequent presenting complaint 90 (76%) followed by lower abdominal pain 11(9.3%) and back pain 5(4.2%). Eight females (6.7%) had gynecological complaint of itchiness was showed in figure 4.2.

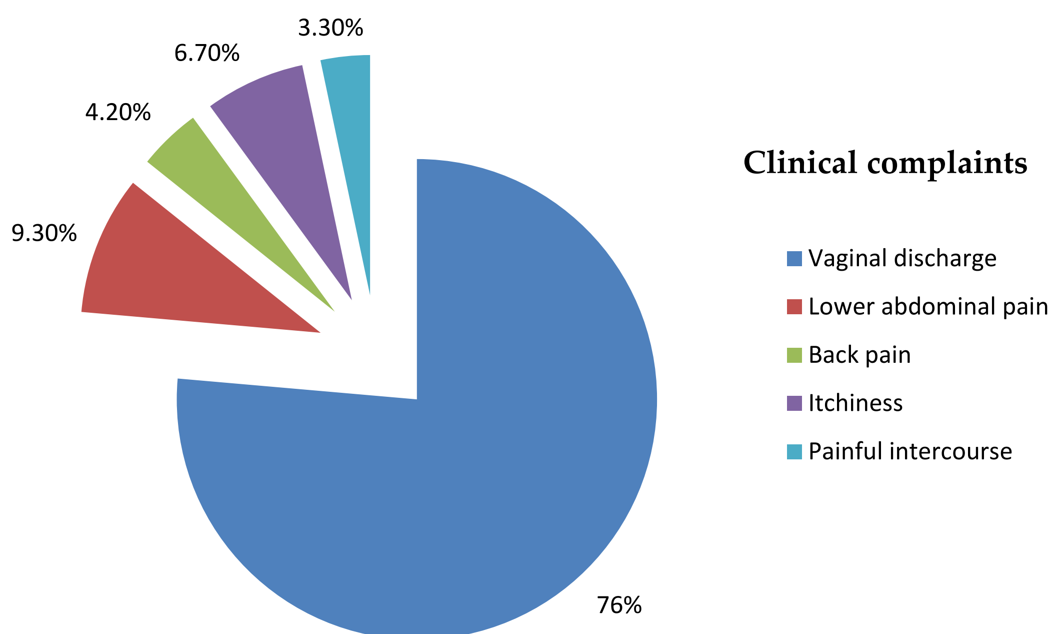


Figure 4.2: Common gynaecological complaints of female patient

Vaginal speculum examination findings showed 54(45.76%) normal cervix, 26(22.03%) suspicious cervix and 38(32.20%) chronic cervicitis respectively. 42(35.5%) Pap smears were reported normal, 33(27.9%) were inflammatory while 18(15.2%) were reported as abnormal pap smear. 11(9.3%) Pap smear was documented as LSIL, 5(4.2%) as HSIL and 9(7.6%) reported as atypical cells (Figure 4.6).

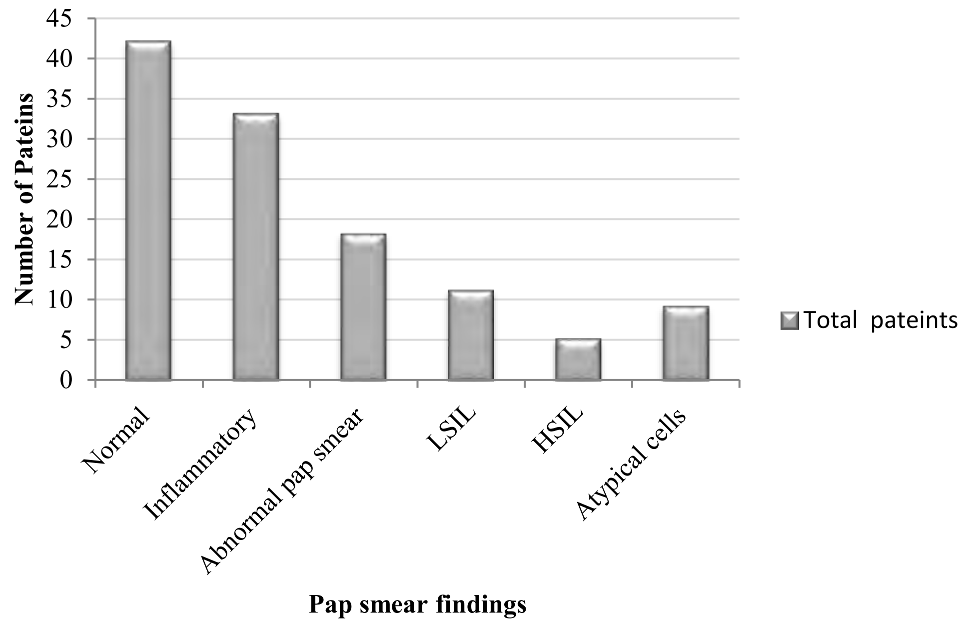


Figure 4.3: Pap smear findings of present study.

Pap smear reported according to Bethesda system (Figure 4.4) and inflammatory, atypical cells, and High grade squamous intraepithelial lesion (HSIL) was showed in figure 4.5 and 4.6 respectively.

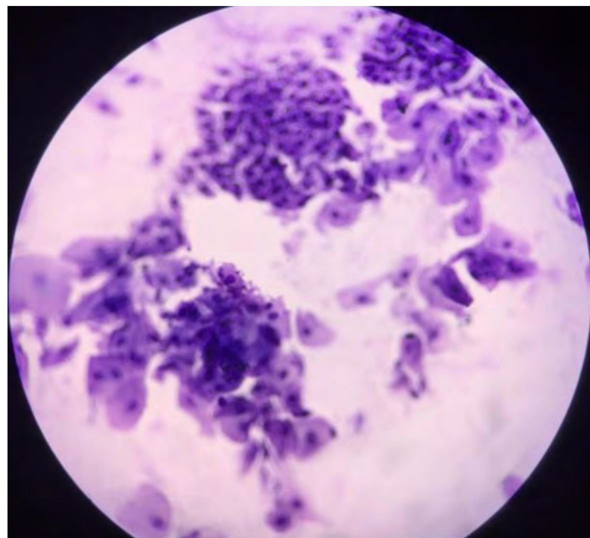


Figure 4.4: Microscopic photograph of normal pap smear showing isolated and clustered basal and parabasal cells.

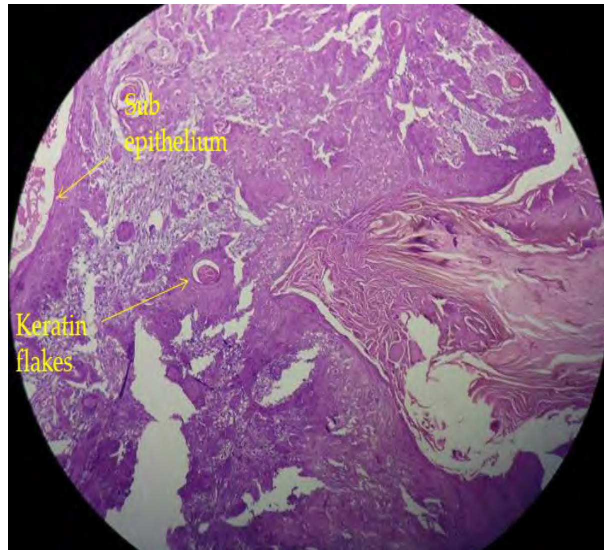


Figure 4.5: Microscopic photograph of invasive squamous cell carcinoma of cervix of large cell keratinizing type (10X power).

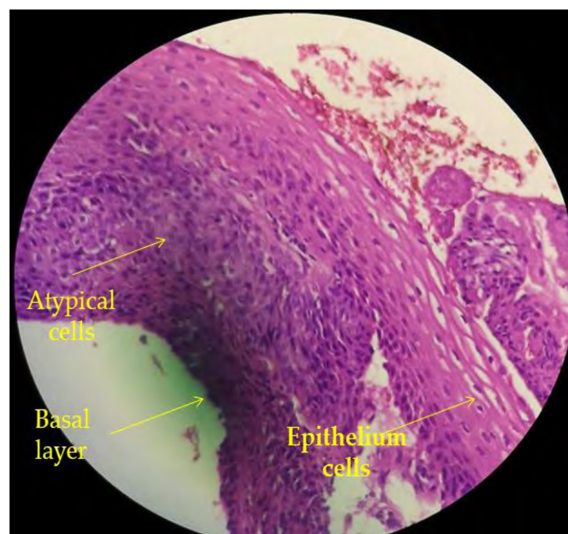


Figure 4.6: Microscopic photograph of high grade squamous intraepithelial lesion HSIL (CIN 2/3). There was proliferation and atypia with increased nuclei cytoplasmic ratio and loss of normal maturation (10X power).

Acetic acid (VIA) was positive in 27/118 (22.8%) patients and negative for 91(77.2%) was showed in table 4.3.

Table 4.3: Visual inspection with acetic acid findings.

VIA	Number (%)
Positive	27 (22.8%)
Negative	91(77.2%)

Age distribution of the 118 patients with positive VIA and Pap smear was showed in table 4.4. Of the 27 females with positive VIA, 9 were between 31-40 years, 12 females were between 41-50 years and further 6 females had age greater than 50 (Figure 4.7). Out of 16 positive Pap smear, 7 females were between 31-40 years and 8 females were between 41-50 years respectively.

Table 4.4: Age distribution of the 118 patients with positive VIA and pap smear.

Age (years)	Total no. of patients	Positive VIA (Total=27)	Positive pap smear (Total=16)
21-30	47	0	0
31-40	41	9	7
41-50	22	12	8
>50	8	6	1

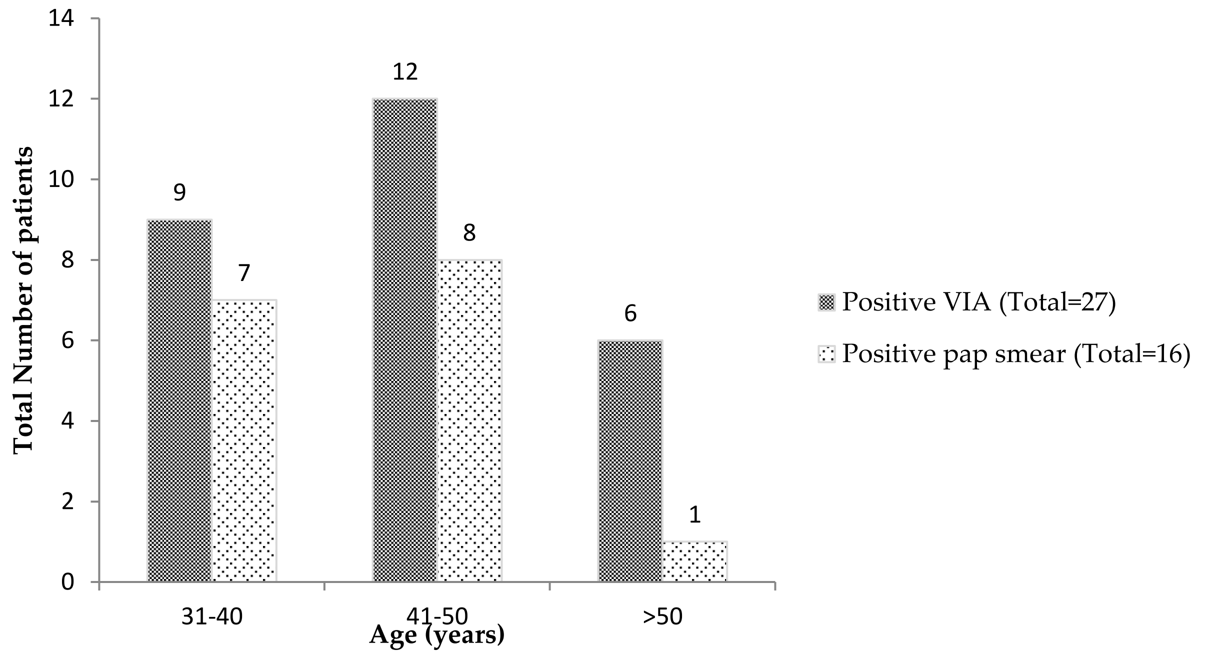


Figure 4.7: Age distribution of the 118 patients with positive VIA and pap smear.

4.3 Phase 3 Results: HPV Screening and genotyping

In present study 105 formalin-fixed paraffin embedded samples with confirmed histopathological characteristics of cervical cancer were studied. According to the International Federation of Gynecology and Obstetrics (FIGO) classification, 92 (87.6%) out of 105 samples were squamous cell carcinoma (SCC), 3 (2.9%) were adenocarcinoma (AD), 4 (3.8%) were adenosquamous cell carcinoma and 6 (5.7 %) were chronic cervicitis.

All biopsy samples were initially subjected to housekeeping β globin gene PCR and 93 (88.6%) were found positive while 12 (11.4%) remained negative. Therefore, a total of 93 DNA samples positive for β globin gene PCR were consequently considered suitable for HPV screening nested PCR using consensus primer (MY09/MY11 and GP5+/GP6+) targeting L1 gene of HPV. HPV DNA was detected in 90 (96.8 %) biopsy samples while remaining 03(3.2%) DNA samples could not be screened as HPV (Table 4.5).

Table 4.5: Frequency distribution of HPV 16 and 18 on basis of histopathological diagnosis.

Histological types	Total Cases	HPV positive cases (n=90)		
		HPV type 16	HPV type 18	Untypeable positive HPV cases
Chronic Cervicitis	6	2	0	0
Well Differentiated Squamous cell carcinoma	49	43	0	4
Moderately differentiated squamous cell carcinoma	38	32	0	3
Poorly differentiated squamous cell carcinoma	5	3	0	0
Adenocarcinoma	3	0	1	0
Adenosquamous cell carcinoma	4	1	0	1
Total	**105**	**81(90%)**	**1(1.2%)**	**8(8.8%)**

4.3.1 HPV-16 and HPV-18 screening PCR

All HPV positive samples (n=90) were further screened for high risk HPV type16 and 18 using respective genotype specific PCR. Among 90 HPV positive DNA samples, 81 (90%) were positive for HPV 16 genotype while 1 (1.2%) was positive for HPV18 genotype and 8 (8.8%) were remained untypeable.

Specific HPV genotype distributions among the diagnosis categories (Table 4.5) revealed that HPV 16 was more prevalent in squamous cell carcinoma.

The age distribution of 90 positive patients cohort was documented to be as follows: mean 52.07 years, range 47, and mode 55years. Table 4.6 illustrated the HPV frequency distribution of samples in present studies in correlation with age. Data about age stratified distributions revealed that the age groups between 41-60 years conceded the highest burden of HPV 16.

Table 4.6: Frequency distribution of HPV genotypes in cervical carcinoma samples in correlation with age.

Age group (Years)	Total cases	HPV positive cases (n=90)		
		HPV type 16	HPV type 18	Untypeable positive HPV cases
21-30	3	0	0	0
31-40	4	1	0	0
41-50	42	35	0	4
51-60	47	41	1	3
61-70	7	3	0	1
71-80	2	1	0	0
Total	**105**	**81 (90%)**	**1(1.2%)**	**8(8.8%)**

4.4 Results of phase 4: DNA sequencing

A total 50 out of 90 confirmed HPV DNA samples were subjected to nucleotide sequencing of L1 gene fragment. Among these samples (n=50), 08 were untypeable, 01 was HPV 18 and 41 were HPV-16 on DNA sequencing. Only 41 out of 81 DNA samples positive for HPV16 on genotyping PCR were included for DNA sequencing due to limited resources.

Phylogenetic analyses classified the study isolates into seven different HPV types based on partial sequencing of L1gene. HPV-16 (n= 41; 82%) was the most frequent type followed by HPV-35 (n=3; 6%), HPV 67 (n=2; 4%), HPV 18 (n=1; 2%), HPV 56 (n=1; 2%), HPV 59 (n=1; 2%) and HPV 73 (n= 1; 2%) respectively.

4.4.1 HPV 16

Phylogenetic analyses confirmed that all HPV 16 study isolates (n=41) constituted a distinct clade and classified them in to two distinct lineages named Lineage I and II was showed in figure 4.8.

Lineage I comprised of 26 HPV 16 isolates from different districts of Punjab, Gilgit, Khyber pakhtoonkhwa and Azad Jammu and Kashmir was showing 97.6% (95.9–98%) nucleotide similarity with HPV 16 prototype strain (accession no. K02718). All isolates in lineage I presented 99.2% (95.8–100%) mean nucleotide similarity with each other and 99.9% (94.8–100%) mean nucleotide similarity with the closely related strains. The most divergent strain in this lineage was PAK-AR21-HPV16 isolated from district Bagh from Azad Jammu and Kashmir with 96.9% nucleotide similarity (Figure 4.9).

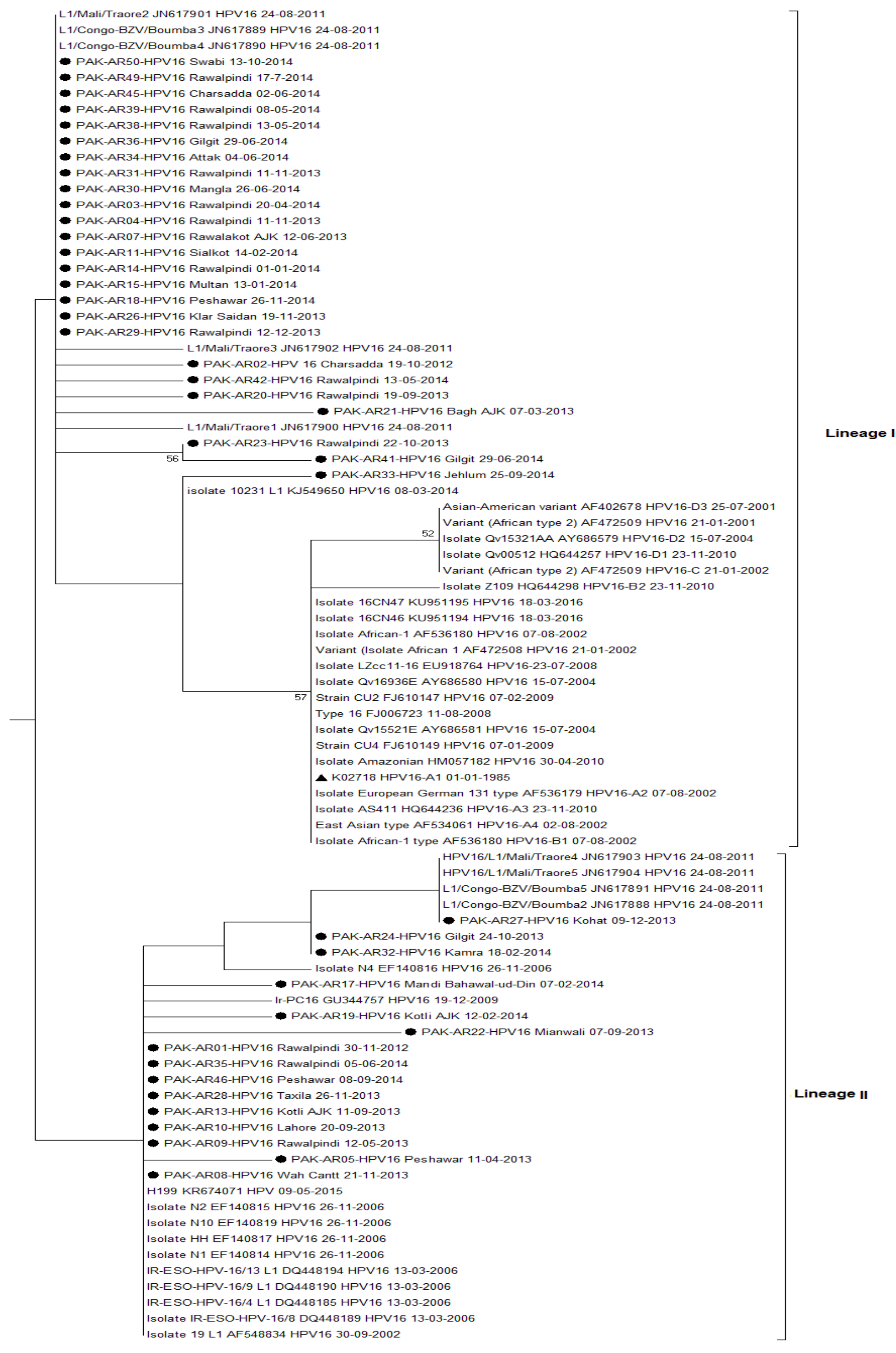


Figure 4.8: Phylogenetic tree of HPV-16 based on partial sequencing of L1gene.

The study isolates and prototype were designated by a '●' and '▲' taxon symbols respectively. A maximum likelihood phylogenetic tree was inferred from Kimura 2-Parameter model in MEGA 5.0 (1000 bootstrap). Numbers on respective nodes indicated the bootstrap values > 50. The scale bar signified the evolutionary distance.

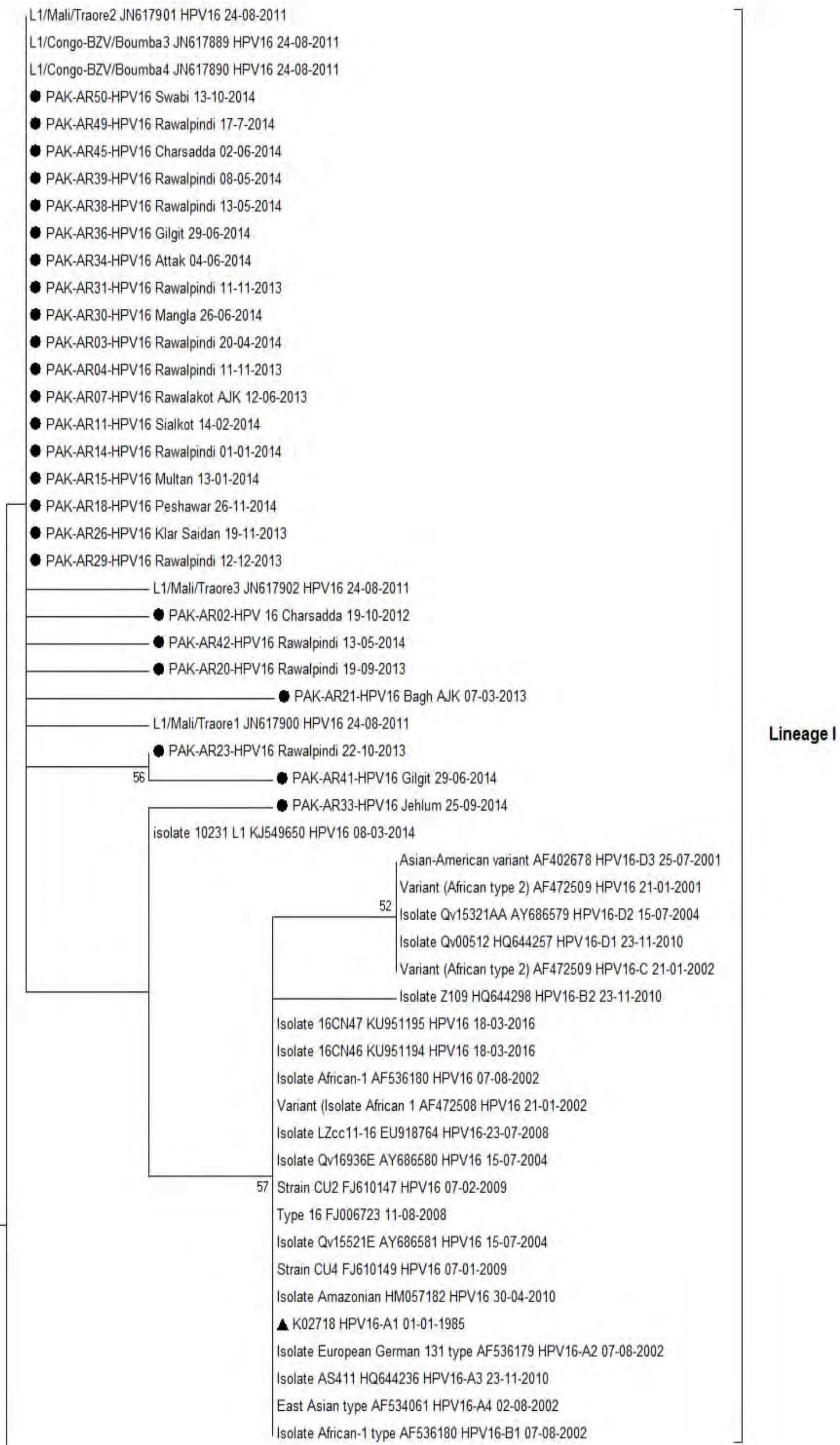


Figure 4.9: Phylogenetic tree of HPV-16 Lineage 1 based on partial sequencing of L1gene

Lineage II contained 15 isolates from different districts of Pakistan had shown in phylogenetic tree with 96.4 % (94.7-96.9 %) nucleotide similarities with HPV 16 prototype strain (accession no. K02718). These isolates had 98.5% (95.8-100%) mean nucleotide similarity with each other and 98.9 % (95.8-100%) mean nucleotide similarity with their closely relative strains. The most divergent strain in this group was PAK-AR22-HPV16 with mean nucleotide similarity 95.8% was showed in figure 4.10.

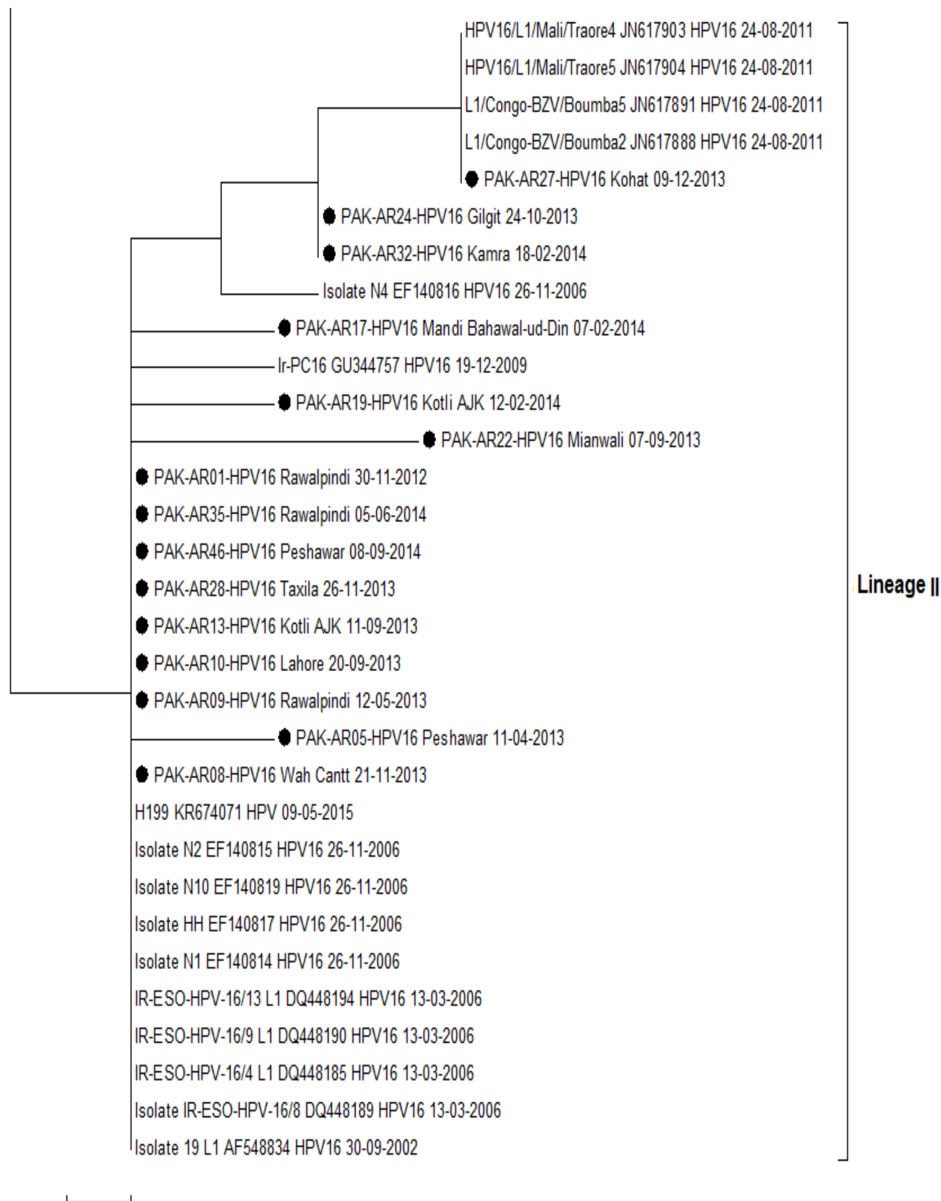


Figure 4.10: Phylogenetic tree of HPV-16 Lineage II based on partial sequencing of L1gene

4.4.2 HPV 35

Similarly, sequencing data confirmed three isolates as HPV 35 revealing 96.4% nucleotide similarity with HPV 35 reference strain (accession no. X74477). Also, they had 99 % (98.6-100%) mean nucleotide similarity with each other and 93.5% (92.5-94%) with their closest relative strain Rw128 (accession no. HQ537727) isolated from infected patient of Rwanda.

4.4.3 HPV18

One isolate (PAK-AR25; Rawalpindi) was confirmed as HPV 18 showed 98.6% nucleotide similarity with HPV 18 prototype strain (accession no. AY262282). The closest relative of this study strain was CU11 (accession no. GQ180787) isolated from infected Thai woman in 2008.

4.4.4 HPV56

One study isolate was confirmed as HPV 56 (PAK-AR47) showed 91% nucleotide similarity with HPV 56 prototype strain (accession no. X74483) and 98.6% with its closely related strain QV26762 (accession no. EF177176).

4.4.5 HPV59

Similarly, phylogenetic analysis revealed one study strains HPV 59 (PAK-AR40), HPV 59 strains had 92.5% nucleotide similarity with their respective prototype. Strains Qv25652 (accession no. KC470261) was found closest relatives of HPV 59 (PAK-AR40).

4.4.6 HPV 67

HPV 67 (PAK-AR44) study strains 95.5% nucleotide similarity with its prototype strains was showed in phylogenetic tree figure 4.11. QV33032 (accession no. HQ537783) was closest relatives of HPV 67 (PAK-AR44).

4.4.7 HPV 73

HPV 73 study strains had 95.6% nucleotide similarity with prototype strains and strain QV00749 (accession no. KF436834) was found closest relatives of HPV 73 (PAK-AR06) respectively was showed in in phylogenetic tree (figure 4.11)

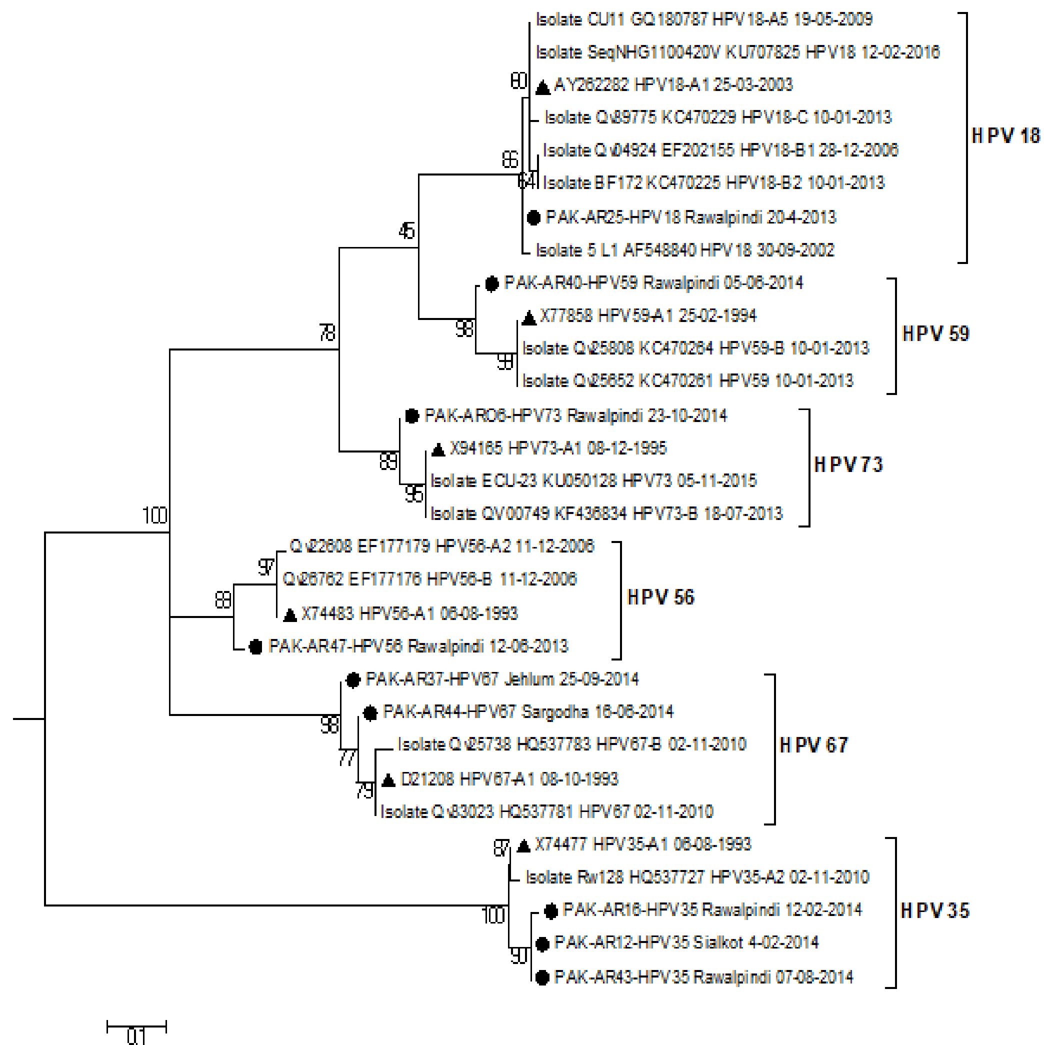


Figure 4.11: Phylogenetic tree of HPV-18, 35, 56, 59, 67 and 73 based on partial sequencing of L1gene.

The study isolates and prototype were designated by a '●' and '▲' taxon symbols respectively. A maximum likelihood phylogenetic tree was inferred from Kimura 2-Parameter model in MEGA 5.0 (1000 bootstrap). Numbers on respective nodes indicated bootstrap values > 50. The scale bar signified the evolutionary distance.

4.5 Result of phase 5: Keratinocyte and fibroblast monolayer cell culture

In this phase the main purpose was to recreate *in vitro* conditions that may be similar to those formed in wound healing. So first of all cell lines, J2-3T3, NIKS and high risk HPV 16 containing NIKS in *in vitro* monolayer culture were established as per protocol mentioned in material and method section. Microscopic images of J2-3T3 (0

hour) at time of seeding and NIKS-HPV16 cell lines after 24 hours were shown in figure 4.12 and 4.13 respectively.

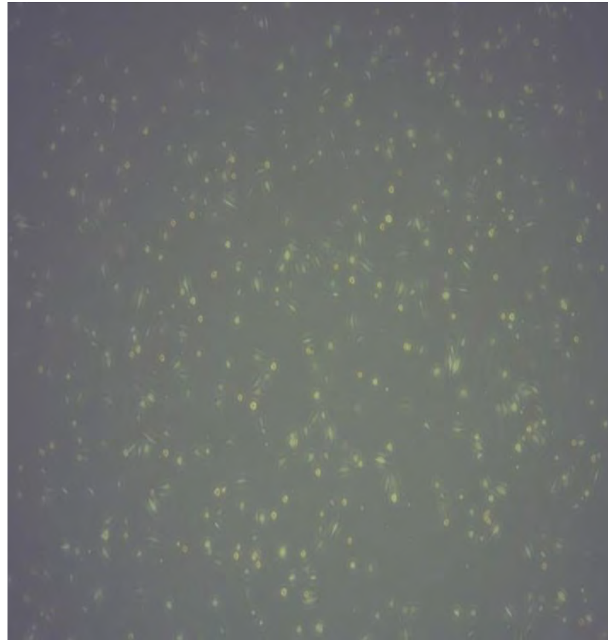


Figure 4.12: Microscopic images of J2 3T3 fibroblast feeder cells which serves as bed for NIKS and NIKS HPV 16 in culture medium. Images were obtained by digitally using 10x magnification objective.

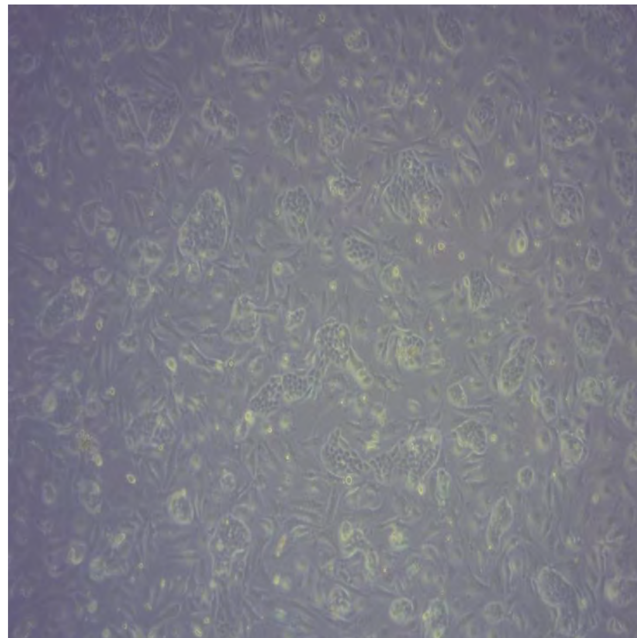


Figure 4.13: Microscopic images of NIKS harbouring HPV 16 in standard culture medium. Images were obtained by digitally using 10x magnification objective.

A standard curve and linear equation was established to check sensitivity and efficiency of each primer set and six 10-fold serial dilutions of template have been prepared which corresponded to recognized number of copies of the template (1 to $1x10^5$). Ct values generated by qPCR for each dilution plotted along y axis and the values of known copy number in each dilution were plotted on x axis showed in figure 4.14 and 4.15 The copy number of each sample was calculated by the standard curves generated for each primer set. It showed that NIKS HPV 16 have greater copy number as compared to NIKS in standard cell culture medium.

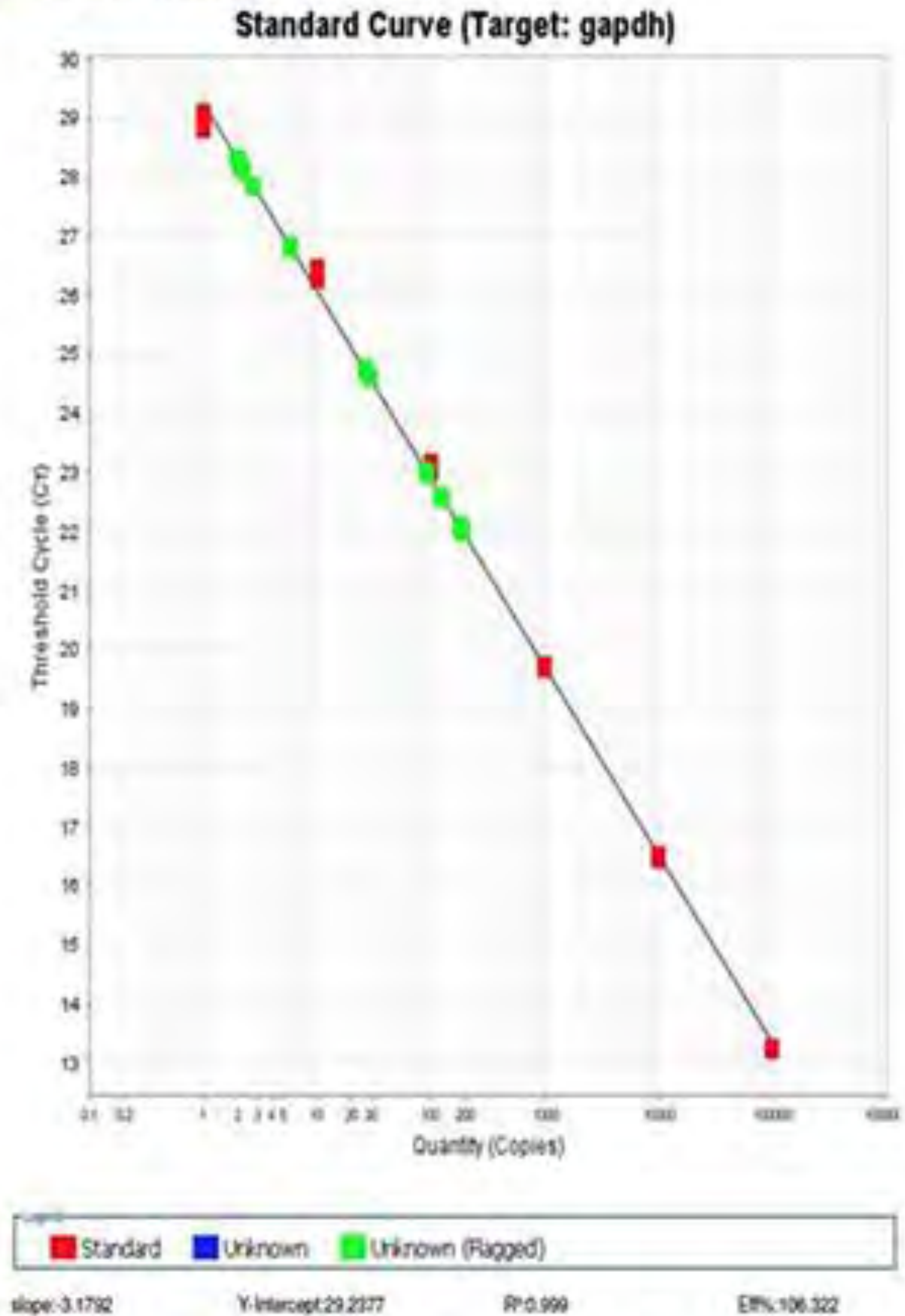


Figure 4.14: A standard curve for GAPDH The slope (m) referred to the efficiency of the primer pairs, where a slope of -3.33 showed a perfect linear relationship between DNA copy number and Ct value and corresponds to an increase of 3.33 Ct for every 10-fold dilution

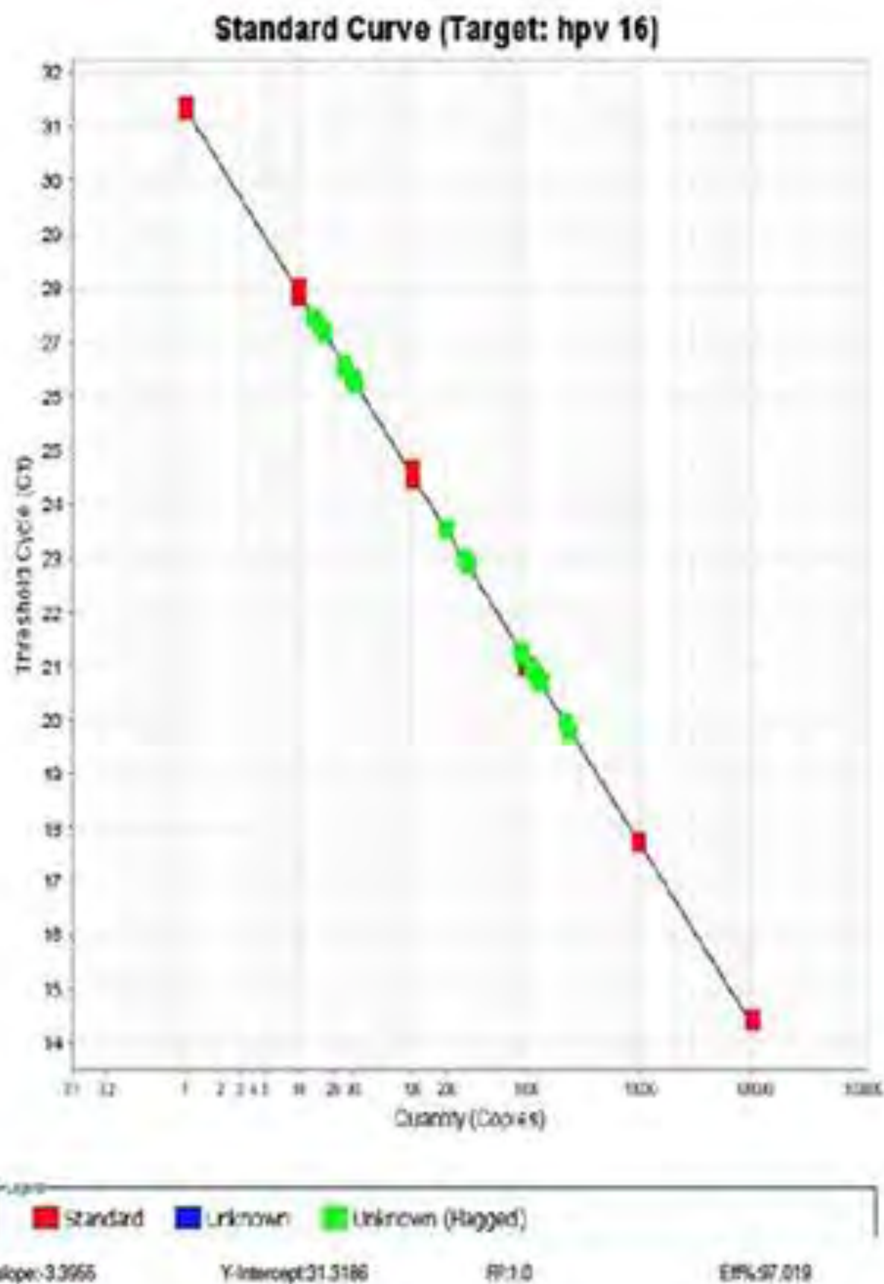


Figure 4.15: A standard curve for NIKS-HPV16. The slope (m) referred to the efficiency of the primer pairs, where a slope of -3.33 shows a perfect linear relationship between DNA copy number and Ct value and corresponds to an increase of 3.33 Ct for every 10-fold dilution.

4.6 Result of phase 6: Monolayer growth assay for determining effect of epidermal growth factor (EGF) on copy number and gene expression

In this phase the impact that wound healing responses may had on formation of lesion due to an epithelial injury were studied. The hypothesis that during wound healing process the occurrence of growth factor at injured site the infected cells may get a proliferative boost enabling them to expand.

Epidermal growth factor is known to be a significant proliferative operator and is utilized in tissue culture to invigorate the NIKS proliferation. Hence, so as to comprehend whether the epidermal growth factor had invigorating impact on infected cells the aim was to reproduce wound healing environment with different concentration s of epidermal growth factors in culture medium. Therefore, present study intended to set up whether a wound healing environment condition may influence the foundation of human papillomavirus induced lesion and altering epidermal growth factor level may stimulate the progression as observed in wound healing *in vivo* when cells were infected with high risk HPV types.

4.6.1 Increase in concentration of epidermal growth factor results in increase HPV 16 copy number

In order to determine that increase in EGF concentration can alter the HPV copy number in a monolayer culture, the concentrations of EGF were increased up to fifty folds as EGF ordinarily contained in a culture media during proliferative period NIKS HPV 16 cell lines to imitate the increase in EGF in post wounding. The experiment was performed in sub confluent conditions in order to avoid the variation in HPV copy number that may happen as an outcome of differentiation of keratinocyte. NIKS harboring HPV 16 were cultured in standard medium up to 80% confluency and re plated in three distinctive concentrations of EGF (10 ng/mL, 100 ng/mL and 500ng/mL) which were comparable with EGF concentrations depicted *in vivo*. It was observed that increase in EGF concentration in culture medium had no impact on morphology of keratinocyte was showed in figure 4.16.

10ng/mL EGF concentration (24 hours)

100ng/ml EGF concentration (24 hours)

500ng/mL EGF concentration (24hours)

Figure 4.16: Microscopic images of monolayer culture at day 1 (24 hours),

Post plating NIKS HPV 16 cells were seeded in equivalent number and cultured using three different concentrations of EGF. Pictures demonstrated the morphology of NIKS HPV16 cells culture with 10ng/mL, 100ng/mL and 500ng/mL EGF. Images were obtained by digitally using 10x magnification objective.

10ng/mL EGF concentration (48 hours)

100ng/ml EGF concentration (48 hours)

500ng/mL EGF concentration (48 hours)

Figure 4.17: Microscopic images of monolayer culture at day 3 (48 hour).

Post plating NIKS HPV 16 cells were seeded in equivalent number and cultured using three different concentrations of EGF. Pictures demonstrated the morphology of NIKS HPV16 cells culture with 10ng/mL, 100ng/mL and 500ng/mL EGF. Images were obtained by digitally using 10x magnification objective.

10ng/mL EGF concentration (72 hours)

100ng/ml EGF concentration (72 hours)

500ng/mL EGF concentration (72 hours)

Figure 4.18: Microscopic images of monolayer culture at day 5 (72 hour),

Post plating NIKS HPV 16 cells were seeded in equivalent number and cultured using three different concentrations of EGF. Pictures demonstrated the morphology of NIKS HPV16 cells culture with 10ng/mL, 100ng/mL and 500ng/mL EGF. Images were obtained by digitally using 10x magnification objective.

NIKS HPV 16 was grown in monolayer growth assay and cells were harvested and pellet was collected for DNA and RNA further analysis. The viral copy number in pellets was estimated by utilizing qPCR approach. The outcomes demonstrated that the quantity of HPV 16 copy number elevated at 500ng/mL EGF was used in culture medium (Figure 4.19). The most noteworthy concentration of EGF prompts increment of virus copy number at 72 hours.

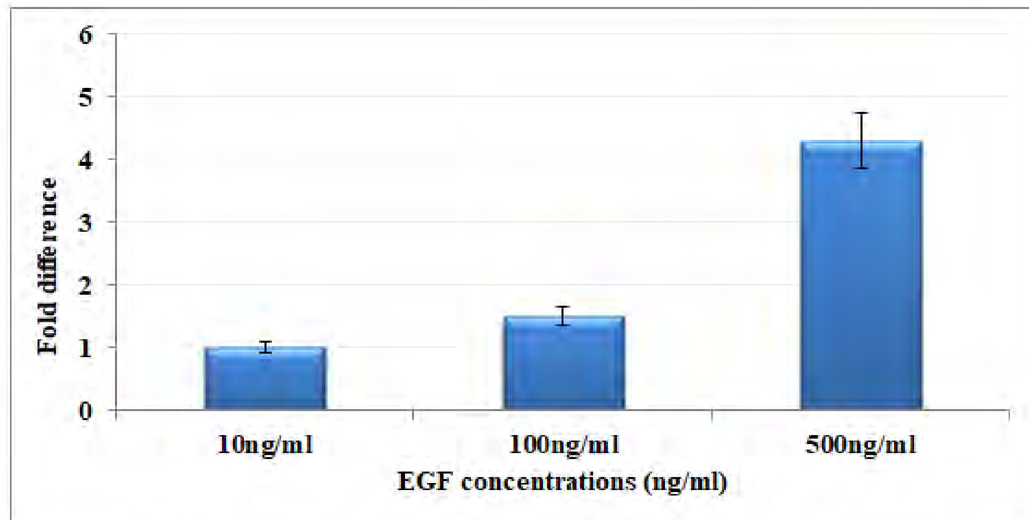


Figure 4.19: NIKS-HPV16 genome copy number at 72 hours compared to HPV16 genome copy number at plating.

NIKS-HPV 16 cells were collected at 72 hours of post plating. After DNA extraction from cell pellet and qPCR was utilized to determine the ultimate copy number of HPV 16 and housekeeping gene GAPDH at initial plating and at post plating time (72hrs). GAPDH copies were utilized to estimate the number of NIKS HPV 16. The bar graph demonstrated the three experiments and range was shown by error bars.

Hence, these findings suggested that the presence of epidermal growth factor at site of infection aids in viral genome replication. The imitation of such effects *in vivo* would be beneficial for virus as keratinocytes having high copy number will undoubtedly proliferate in latter phases of formation of lesion.

In present study a high concentration of EGF medium was used in monolayer growth assay to determine the impact of wound healing response on the amplification of viral

genome ad on viral gene expression. As demonstrated before, utilizing an EGF enrich medium brought about an increase in NIKS HPV 16 copy number by 72 hours.

Hence, in order to know the possible long term impact of EGF treatment on copy number of NIKS HPV 16, the quantity of viral genome copy number after treatment with EGF for 96 hours was also assessed. The test meant to set up whether changes in the genome copy number were sustained when 10ng/mL EGF lower concentration was restored in the culture medium, as would occur in the days following epithelial injury.

Therefore, NIKS HPV 16 cell were cultured using three different concentrations of EGF (10,100 and 500ng/mL) in seven six well plates so as to acquire triplicate wells for each time point and left for 72 hours. A separate NIKS HPV 16 cell monolayer culture in 10ng/mL was established which was utilized as “control”. Following 72 hours the initial set containing three plates harvested and DNA was extracted to determine copy number. So as to reproduce the reclamation of ordinary EGF after epithelial injury the remaining three plates were further kept for 96 hours in standard complete medium that is 10ng/mL and later cell pellet was collected and DNA was extracted was shown in figure 4.20. This proposed even a small exposures to increase concentration of EGF may trigger a mechanism that raises the viral copy number in cells which were infected.

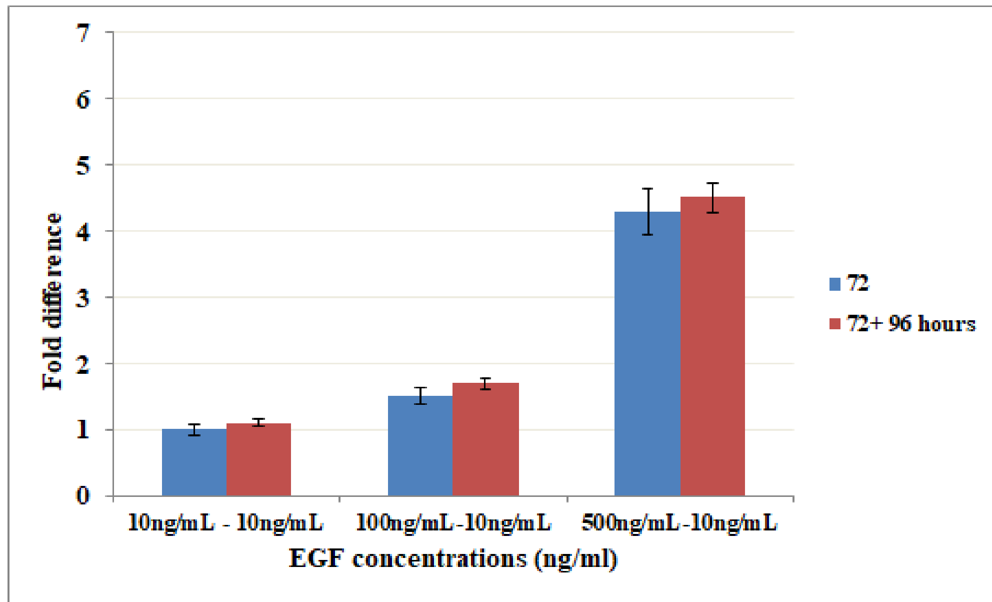


Figure 4.20: Evaluation of change in copy number of NIKS HPV cell 16 population.

NIKS HPV 16 was cultured in three different concentrations of EGF (10, 100 and 500ng/mL). Following 72 hours the monolayers were further cultured in 10ng/mL EGF medium for 96 hours. The copy number assessed at 72 hours and 96 hours normalized with copy number measured at plating point and bar graph was plotted that showed average of three experiment and error bars represents the range.

4.6.2 Increasing EGF concentration does not produce a proliferative effect in monolayer cultures

NIKS HPV 16 cell population treatment with high concentrations of EGF lead to increase in copy number of HPV 16. In order to determine whether the increase concentration of EGF in culture medium could likewise prompt to increase in cell proliferation a specific monolayer growth assay was performed. Hence, NIKS HPV 16 cells were cultured for measurement of the rate of proliferation. Triplicate wells were used for each concentration and cells were harvested at regular intervals (every 48 hours) for 7 days.

The results revealed that HPV 16 cells cultured in high concentrations of EGF 100ng/mL and 500ng/mL NIKS HPV produce a more noteworthy number of cells at day 5 and 7 as compared to NIKS HPV 16 cultured in low concentration of EGF medium (10ng/mL). Furthermore, the cell number of NIKS HPV 16 counted at day 3

was lesser than cell cultured in standard EGF medium (10ng/mL). In spite of fact that the cell number was deliberately fine, hence medium enriched with growth medium permitted the NIKS HPV 16 cells to proliferate more proficiently at post confluency. Hence NIKS HPV 16 copy number rises when presented to high EGF concentration but it does not necessarily increase cell proliferation (figure 4.21).

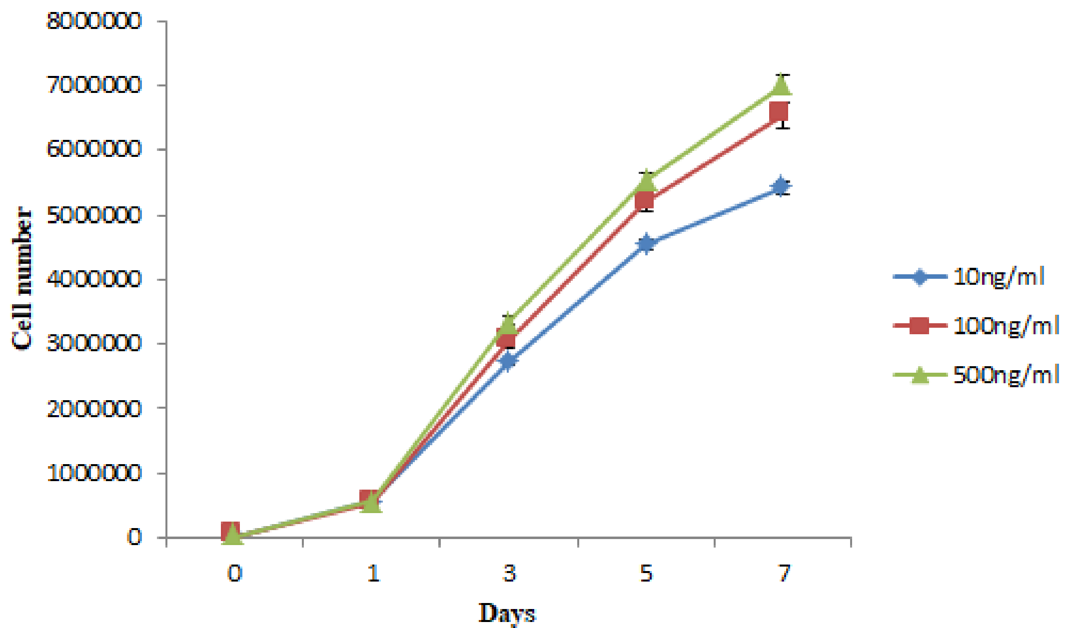


Figure 4.21: NIKS-HPV16 monolayer growth assay. Cells were cultured for seven days in different concentration of EGF (norm=10ng/ml; +10=100ng/ml; +50= 500ng/ml) in a medium.

The cell number of six different wells/time points was obtained using a coulter counter. Cell numbers were averaged for each time point and plotted in a graph.

4.6.3 NIKS-HPV16 cells in EGF high concentration have different gene expression

Since in wound healing EGF concentration varies hence HPV 16 oncogene alternative splicing in monolayer model framework was used in present study. Therefore, specific primer sets were used to validate the oncogenic proteins proportions delivered from viral episomes. When NIKS HPV 16 were presented to high convergence of epidermal growth factor 100ng/mL and 500ng/mL high level of E6 full length mRNA was detected.

The mRNA expression level of HPV 16 transcripts (E1,E2,E4, E6 ,E6* ,E7 and E8) was done quantitative RT-PCR approach with total RNA extracted from NIKS HPV 16 culture media enrich with EGF in monolayer growth assay with three distinct concentrations of EGF 10ng/mL,100ng/mL and 500ng/mL for 72 hours. Moreover GAPDH was utilized as a reference gene. The data presented in table was obtained from mean values of independent experiments repeated thrice was shown in table 4.7.

Utilizing qPCR, an average of 2-3 fold increase in expression of E6 transcript was observed when cells were exposed with 100ng/mL and 500ng/mL EGF contrary to the cells in standard EGF medium (10 ng/mL) was showed in figure. EGF has significant impact on the expression of E6*.

It was observed that 100ng/ml and 500ng/ml also have increase E7 and E4 transcript. However, no significant variation was measured in the level of mRNA expression of E8 following increase EGF stimulation.

Table 4.7: NIKS-HPV16 cells exposed to high concentration of EGF showed variations in the splicing pattern of mRNA.

EGF Conc. (ng/ml)	Days	RNA copies per 1000 cells						
		E4	E8	E2	E1	E6*	E6	E7
10	Day 1	2900	194	462	223	4178	6538	1833
	Day 2	4344	235	1205	669	11800	14120	13352
	Day 3	9941	601	1536	825	17500	31683	28076
100	Day 1	4989	380	524	262	3000	9000	7800
	Day2	10625	559	1423	705	11923	15737	19750
	Day3	14115	740	1650	995	19411	38823	23846
500	Day 1	4735	659	560	240	8033	35294	9508
	Day 2	11340	712	1874	874	18000	44791	22058
	Day 3	33060	946	1955	1500	21538	63134	55104

The mRNA expression level of E6 mRNAs was determined by performing quantitative RT-PCR approached with total RNA extracted from EGF-stimulated NIKS-HPV16 cells cultured in monolayer for 3 and 5 days, using 10ng/ml, 100ng/ml and 500ng/ml of EGF (Figure 4.22). The fold increase in the expression level was standardized to the expression level measured at seeding point. GAPDH expression was used as reference gene. Expression levels of E6 demonstrate increment at day 5 in 500ng/ml respectively was showed in figure 4.23.

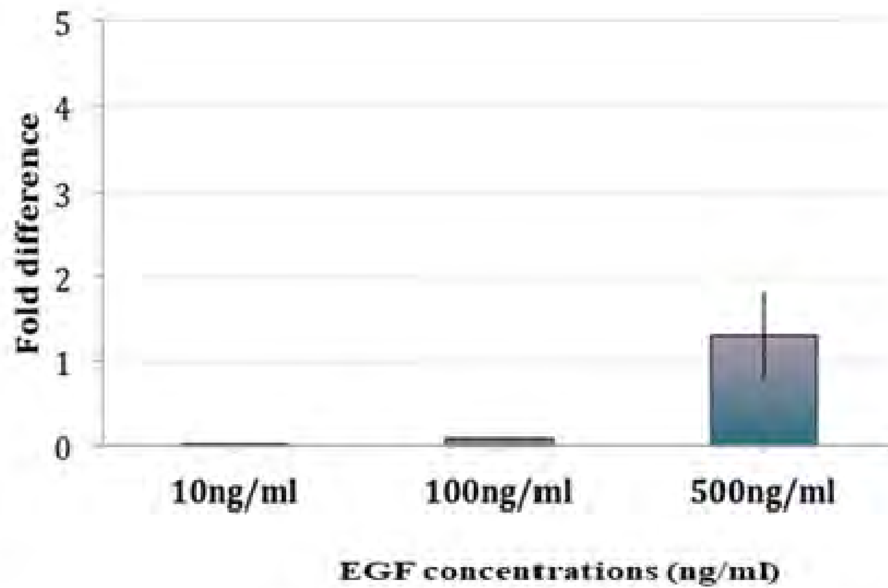


Figure 4.22: Quantitative real-time RT-PCR analysis of E6 transcripts in NIKS-HPV16 populations.

Bar graph showed increased expression of E6 when concentration of EGF was 500ng/mL at day 3. The data was generated from the mean values of three independent experiments and the standard deviation (±) was presented as error bars.

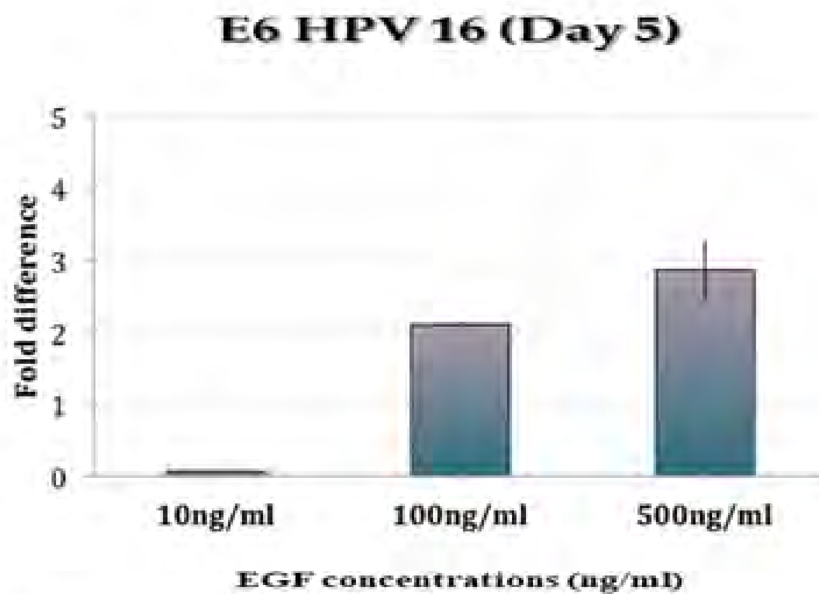


Figure 4.23: Quantitative real-time RT-PCR analysis of E6 transcripts in NIKS-HPV16 populations.

Graph demonstrated increase expression of E6 full length at high concentration of EGF in culture medium at day 5. The data was generated from the mean values of three independent experiments and the standard deviation (±) was presented as error bars.

5. Discussion

Cervical carcinoma influences females in their reproductive years. Carcinogenic transformation of HPV infection to intrusive malignancy is a very long process and it gives adequate chances to detect the stage of disease, its diagnoses and treatment. Therefore, cytological screening is a major secondary prevention approach for early detection (Basu *et al.,* 2018; Kadam *et al.,* 2018). In low-middle income countries low coverage of screening facilities for cervical cancer has been ascribed to personal and structural barricades. Personal barricades comprises of less information concerning cervical malignancy, screening practices, preventions and acumens to socio-religious convictions. A structural barricade involves inaccessibility of screening programs, inadequate resources (material and financial) and poor health-care services (Denny *et al.,* 2006; Morema *et al.,* 2014; Finocchario-Kessler *et al.,* 2016).

This study focused on measuring cervical cancer screening aspects in Pakistani women and evaluated the relationship between screening and the social and demographic aspects. Numerous studies proposed that knowledge concerning carcinoma and Pap smear test are influenced by different factors including age, education, husband's education, parity and household income (Al-Shaikh *et al.,* 2014; Mulhim *et al.,* 2014; Du *et al.,* 2015; Strohl *et al.,* 2015).

All female patients in the present study were married and the mean age is 35.78 ± 9.8 years. Many studies have documented that the average age of female to develop cancer of cervix lies between 40-50 years old. However, its precursor lesion develops earlier (5-10 years) (Saraiya *et al.* 2013; Shanmugham *et al.,* 2014; Maleki *et al.,* 2015b). Hence, the existing study also highlights that married females should have one screening test at age of 35years old as this cervical cancer screening play a key role in diagnosis and disease management.

In present study, mean age at first pregnancy was 18.06 ± 9.77 years old. Previous data showed that birth of child at very early age could influence the risk of cervix carcinogenesis. This susceptibility may be due to steroidal hormone influence on human papillomavirus infection, host immune response to infection in puberty and biological immaturity in adolescence (Elson *et al.* 2000; Louie *et al.* 2009).

The study also assessed the influence of some significant independent variables like; education, qualification and employment status of husband, family income, area, parity, smoking on screening. In this study most of patients are from Rural areas 68(57.62%) belong to low (58.5%) income socio economic status. The study found in agreement with other local and international studies that poor socioeconomic status and lack of education, smoking poor hygiene has positive association with progression of disease (Ali *et al.* 2010; Das *et al.* 2013; Jbeen *et al.* 2013). Hence, higher level of education is positively associated with better knowledge about cervical cancer screening (Aldohaian *et al.,* 2019b). Vaginal discharge is a very common gynecological complaint. The present study showed that 90(76%) female patients have clinical complaint of vaginal discharge. This validated with the findings of various global investigations (Samanta *et al.,* 2011; Yasmin and Mukherjee 2012; Salih *et al.,* 2017).

This study revealed that 118 of female patient had undergone a Pap test. Among them dysplastic changes were observed in 16, in which (11 (9.3%) showed LSIL(low grade squamous intraepithelial lesion) and 5(4.2%) patients have HISL (high grade squamous intraepithelial lesion). Several local studies have documented a relation between cervical malignancy and abnormal cells in cervix detected on Pap smear (Khattak *et al.,* 2006; Haider *et al.,* 2013; Khan Inamullah *et al.,* 2013).

Detection rate of cervical dysplasia in VIA is 22.8 % in our study. Numerous studies had documented 1.7 % to 29% positive VIA cases (Tayyeb *et al.,* 2003; Bomfim *et al.,* 2005; Albert *et al.,* 2012). This variability in rate is because of various criteria utilized in different investigations because of absence of standard measures for positive outcomes. Our finding in the present study is the VIA highest positivity rate is in the age group of 40-50years. Results of the present study are similar with other studies that VIA and Pap smear positivity frequency increases with the age (Gravitt Patti E *et al.,* 2010; Arun *et al.,* 2018).

Pakistan is an Islamic state, it assimilate religion in all areas of life including law, culture and society. But prevalence of a large number of myths, socio-cultural and religious taboos has adversely affected the cervical cancer screening (Shafiq and Ali 2006; Khan Saeed *et al.,* 2007a). False elucidation of Islamic convictions prompted

42% females to assume that "if cervical cancer is piece of my fate having a screening test can't forestall it". Islam urges Muslim to perform self-care and seek out treatment. It was narrated that the Holy Prophet Hazrat Muhammad (peace be upon him) said: "seek treatment, O salves of Allah! For Allah doesn't create any disease but he also creates with it the cure except old age" (Salman 2012; Aldohaian *et al.* 2019b).

Several studies suggested the role of self-sampling technique as many women may incline toward a test that can be performed in privacy by the woman herself in her home to avoid discomfort or embracement with in the medical staff (Martin 2000; Gok *et al.,* 2010; Singla and Komesaroff 2018). Numerous studies recommended that media should play its key role in disseminating information and awareness about cervical cancer and importance of Pap smear test (Cetisli *et al.,* 2016; Aldohaian *et al.* 2019b).

Globally, Pakistan is sixth most populous state, with an estimation of more than 195.390 million people as indicated by population census organization of Pakistan. Cervical cancer is among common cancers in the country with 8.4 % incidence and 7.2 % death rate (Ferlay *et al.* 2014). The role of viral infection into advance progression of carcinoma of cervix in female residing Pakistan has not been broadly comprehensively addressed because of lack of awareness of people on sexually transmitted diseases (STDs) and the stigma attached to sexual diseases in country (Khan Saeed *et al.* 2007b; Gul *et al.* 2015). There are few studies that provide little knowledge of HPV in the country. Furthermore, these studies focused largely on two cities Karachi and Lahore (Khan Saeed *et al.* 2007b; Raza *et al.,* 2010; Yousuf *et al.,* 2010; Siddiqa *et al.,* 2014; Loya *et al.* 2016). On the other hand current study explored the frequency of HPV in wider area of country including Punjab, Khyber Pakhtunkhwa, Gilgit Baltistan and Azad Jammu and Kashmir. The present study provides the information on frequency and distribution of various genotypes in cervical carcinoma patients in Pakistani women (Villa and Denny 2006b).

Conventional PCR ,Real time PCR procedures and hybrid capture and micro arrays are various molecular diagnosis methods for detection of HPV (Tawe *et al.,* 2018). For HPV diagnosis PCR method is considered as "gold standard", but unfortunately, rarely applied in Pakistan for HPV diagnosis. HPV DNA detection by MY09/MY11

and GP5+/GP6+ primer sets is a sensitive metho,. (Haws *et al.,* 2004) both targeted viral genome's L region .(Gene ID: 1489082) This reulsts in detection of a wide range of HPV genotypes (Gravitt PE *et al.,* 1998; Gravitt PE *et al.,* 2000; Hubbard 2003; Kösel *et al.,* 2003; Organization 2010).

Molecular methods like PCR used for HPV detection target the L1 gene, up-stream regulatory region (URR) or the E6/E7 genes which are constantly retained in infected and neoplastic cells. HPV DNA testing by PCR has become a standard and non-invasive method for identification of cervical HPV infection. This method precisely identifies HPV DNA with distinctive low concentration in cells from histocytological screening of cervical lesions (Gravitt PE *et al.* 1998; Gravitt PE *et al.* 2000; Hubbard 2003; Kösel *et al.* 2003; Organization 2010).

In current study nested PCR successfully detected HPV infections in 90 (96.8 %) biopsy samples while 03 (3.2%) remained negative. This nested PCR amplified HPV DNA samples effectively. However, proportion of negative cases is due to the fact that extraction of DNA from FFPE tissues remains a challenge. There are different studies that described that these problems may be due to traces of xylene during the extraction procedures (Shi *et al.,* 2004) prolong digestion with proteinase K (Shedlock *et al.,* 1997) and the fixation methods i.e fixatives and process of fixation may results in impairment of DNA (Baay *et al.,* 1996; Srinivasan *et al.,* 2002; Plenat *et al.,* 2006; Gilbert *et al.,* 2007). Moreover, our study proposed to use this nested PCR method for routine screening of HPV infections which is easy, cost effective and can be valuable to resource limited settings.

This study proposed that HPV16 is the most prevalent genotype in country. These results are in agreement with the local studies (Siddiqa *et al.* 2014; Gul *et al.* 2015; Loya *et al.* 2016) and studies carried out in other countries of a similar religious and cultural beliefs, such as Indonesia (Schellekens *et al.,* 2004), Saudi Arabia (Alsbeih *et al.,* 2011) and Iran (Shahsiah *et al.,* 2011). The histopathological diagnosis correlated with genotypic distribution of HPV revealed that there is a high association of squamous cell carcinoma with HPV16 which is in concurrence with earlier studies (Bhurgri *et al.,* 2007).

Age plays an imperative role in cervix malignancy. Incidence of HPV in healthy women varies with age (Franceschi *et al.* 2006). Highest burden of malignancy is between 51-60 years than 41-50 years in this study was consistent with worldwide age distribution data for cancer of cervix. The increase in HPV frequency in old age could be attributed to human estrogen and progesterone level changes affect the cervical epithelial metaplasia process and support the HPV replication; Moreover, the declining of body immune function leads up to decrease the ability of HPV clearance and increase the susceptibility to an HPV infection (Zhou *et al.* 2017).

Therefore, it is important to carry out the detection of HPV infection in the elderly women in order to prevent the occurrence of cervical cancer. The proportion of cervical disease in Pakistan is lesser than in different western nations, however higher rate of mortality is credited in view of the late presentation of cervical malignancy due to poor knowledge of screening and social restrictions on all sex related affairs (Bukhari *et al.,* 2012). Moreover, screening and prevention programs of HPV for women in Pakistan are not yet established.

Present study showed that 8 (8.8%) of positive HPV were remained untypeable on genotype specific PCR for HR-HPV16 and 18. However, these samples were positive for HPV DNA on consensus PCR that revealed they may have some other genotypes other than HPV16 or 18 or have some variations at the primer binding site (Schenk *et al.,* 2009). However, DNA sequencing successfully typed these samples. Numerous human papillomavirus genotyping assays have been described, each with its own advantages and disadvantages that often limited resolution and specificity (Lindh *et al.* 2007; Tardif *et al.,* 2013).

DNA sequence analysis may validate the human papillomavirus genotyping. Studies suggested that DNA sequencing is useful tool for accurate HPV genotype identification. Also it can be helpful in unveiling novel human papillomavirus subtypes (Lee Sin Hang *et al.,* 2014). L1 gene is profoundly conserved and the degrees of divergence in L1 play important role in papillomavirus classification in genera, species, types and subtypes (De Villiers *et al.* 2004). In current study, sequencing and phylogenetic analysis successfully identified seven HPV genotypes with most frequently found HPV type, 16 followed by HPV35, 18, 56, 59, 67 and 73.

Uptill now according to best of our information there has been a few epidemiological research on HPV in different female age groups in the country.

Moreover, DNA sequences of HPV types 35, 56, 59, 67 and 73 were not reported earlier in Pakistan. This data correspond with earlier studies and support that HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, 68 and 73 genotypes have important role in cervical carcinogenesis (Munoz *et al.,* 2004; Bertelsen *et al.,* 2006). With regard to HPV56 only one HPV type (2%) was analyzed in this study. HPV56 accounts for a small proportion (<1%) of cervical carcinogenesis glo bally (de Sanjose *et al.,* 2010). In Asia HPV56 prevalence is remarkably higher in china, however limited data is available on HPV56 variants and there is need of sequence variation to be elucidated among Asian population (Zhai and Tumban 2016; Jing *et al.,* 2018).

In spite of the introduction of prophylactic vaccines for human papillomavirus associated infections and cervical carcinoma, it is evaluated that this won't drastically influence the disease burden for 30 to 50 years (Stanley Margaret *et al.,* 2012). However, many investigations focused on studying HPV 16 and 18 types, it is essential to build understanding of various high risk HPV types beside this pursuing additional understanding that how human papillomavirus virus destabilize the molecular pathways. Developments in such areas will improved strategies for treatment of disease and are significant to complement on going disease management. Moreover, the learning of high risk human papillomavirus associated disease not only related to cervix cancer but also esophagus and oropharyngeal region. The last part of this study focuses on how high risk human papillomavirus form benign lesion which may progress to malignancy. To achieve this, a model frame work was established in which we can imitate the environment in which human papillomavirus develop a lesion. Different experiments were performed to understand the human papillomavirus dynamics and factors responsible for increase in copy number and proliferation as occur during the underlying period of human papillomavirus infection in wound healing. Several studies had investigated the role of the environment of the basal cell in the formation of lesion due to HPV and specifically considered that formation of epithelial injury in basement membrane permits virus attachment (Kines *et al.* 2009).

Therefore, following this *in vitro* conditions were recreated that would be comparable to those made during wound healing process. Initial focus was on understanding the dynamics of high risk human papillomavirus containing normal immortalized human keratinocytes (NIKS) *in vitro* in monolayer culture medium. Data showed that NIKS harboring HPV 16 (infected cells) have more ability to migrate and proliferate than normal NIKS cell (uninfected cells) in monolayer culture medium. This finding demonstrated that such phenotypes would give advantage to infected cells during wound healing process. Such proliferation would enable infected cell to exceed its neighboring uninfected cell which leads to infection or expansion of lesion after injury. This is in accordance with past studies that utilized cancer cell lines which expressed high risk human papilloma virus (Valencia *et al.* 2008; Yeung *et al.* 2011; Wechsler *et al.,* 2012).

Current study also extended to determine the extra impact that enriched growth factors condition may deliver on the behavior of infected cells. Several studies have demonstrated that in normal wound healing process cells have not much area to multiply but they are triggered by the occurrence of several growth factors (Schultz *et al.,* 1991; Cribbs *et al.* 2002). Under ordinary conditions the presences of growth factors play a dynamic role in multiplication of cells at site of injury. Present study has tried to determine the effect of epidermal growth factor (EGF) on viral replication and gene expression. Hence, current study findings showed that increases in EGF concentration will also increase viral copy number in high risk HPV types. Furthermore, it revealed that the treatment with EGF had no important effect on NIKS HPV 16 proliferation.

Present data also revealed that EGF impact on genome replication was better supported in high risk HPV types and hence it proposed that mechanism of viral gene replication was interfering by external stimuli and also assessed the impact of viral gene expression due to increasing concentrations of EGF. A study has demonstrated the epithelial growth factor inclination is combined with joining of E6 mRNA (Rosenberger *et al.,* 2010).

After epithelial injury the growth factors occurrence raised instantly (Sheardown and Cheng 1996; Wilson *et al.,* 1999). Keratinocytes growth is facilitated by these family including fibroblast growth factor, insulin like growth factor. (Werner S. and Grose

2003b). Epidermal growth factor level in tears demonstrated a noteworthy increase after corneal damage. Various EGF protein and mRNA in corneal damage is raise approximately seventy folds promptly after injury and within 72 hours come back to basal level (Brightwell *et al.,* 1985; Wilson *et al.* 1999).

This study also showed that growth factor variation in concentration create an impact on infected cells. This was achieved by varying EGF concentrations (10, 100 and 500ng/mL) in medium to recreate wound healing like conditions. It also suggested that increased in EGF concentration can increase the viral copy number. Moreover, keratinocytes having more copy number have high ability to persevere and proliferate in different stages of viral lesion formation. It may be due to NIKS harboring HPV16 were stimulated by EGF to make increase number of viral episomes and the viral genome proficiently neutralizes the host cell mechanism that control exogenous DNA replication. This was in agreement with previous finding that if the environment is growth factor enriched environment some how aids to the extension of HPV infected cells during the healing process of wound (Collins *et al.,* 2005).

A correlation was established between different levels of epidermal growth factor and various human papillomavirus genome transcripts in present study. It yields some interesting findings that using different concentrations of EGF in monolayer growth assay, NIKS HPV 16 population when cultured in medium having higher concentration of EGF not only increased the copy number but it also increased the transcript level of viral oncogene E6 and E6*. It means higher dosage of epidermal growth factor aids in formation of E6 splicing form. Studies have shown that epidermal growth factor in culture medium can transform the splicing pattern of E6 and E6* (Collins *et al.* 2005) and differential splicing can further cause polycistronic mRNA regulation (Schwartz 2008; Jia *et al.,* 2009).

However, early mRNA alternative splicing prompts translation of various functional proteins like E6, E6* and E7 (Grassmann *et al.,* 1996; zur Hausen H. 2002). Extracellular stimuli also regulate alternative splicing as various pathways have been found that control this procedure. The system in which these factors apply functions involved splicing factors activation and suppression (Blaustein *et al.,* 2007). We have demonstrated that increase EGF level involved in E6 and E6* alternate splicing in

NIKS HPV 16 cell *in vitro*. Furthermore we have also investigated E2, E4, E7 and E8, whether splicing patterns of other open reading frames have changed by the increase concentration of epidermal growth factors. The polycistronic E6 and E7 mRNA splicing produce a new open reading frame E6*mRNA and this is specifically described in high risk human papillomavirus (Zheng Z. M. and Baker 2006b).

In present study E6 full length were more abundant as compared to E6* and previous data showed that E6* open reading frame species are the most inexhaustible transcripts in high risk human papillomavirus associated lesions and carcinomas (Bohm *et al.,* 1993). However, few biochemical differences in regard to various E6 isoforms have been analyzed and sparse data is available on *in vivo* regarding its function inside epithelium and alternate splicing regulation (Schwartz 2008). Hence present *in vitro* study revealed that the increased concentration of epidermal growth factor has ability to interfere with the regulation of oncogenic proteins of human papillomavirus and in wound healing process virus require increase E6 to stop apoptosis so it can increase its copy number and it can be achieved by high concentrations of growth factors at infection area. Consequently all findings suggested that increase concentration of epidermal growth factors has an impact on copy number and gene expression of high risk human papillomavirus.

6. Conclusions

This study generated adequate and reliable data that contributes in understanding of prevalence, clinical outcomes and molecular epidemiology of human papillomavirus circulating in Pakistan. Moreover, it also provides additional information regarding epithelial wound healing *in vitro* model system. Following conclusions can be drawn from the current research projects:

- Majority of circulating strains of HPV in the setting are commonly prevalent strains also reported from different countries.
- Screening plays a vital role in early diagnosis and disease management. Therefore screening programs should be established at national level.
- VIA is an effective method for immediate detection of cervical malignancies as compared to Pap test.
- HPV 16 was more prevalent as compared to HPV 18 in squamous cell carcinoma while HPV18 was found in adenocarcinoma.
- Prevalence and phylogenetic analysis of HPV 35,56,59,67 and 73 were first time reported in Pakistan. This is helpful in understanding the changing pattern of HPV infection and disease association.
- Cervical cancer mortality rate is higher in Pakistan due to late presentation of disease.
- In the wound healing model, EGF administration, not only the viral copies were increased HPV16 genome containing NIKS, but also the transcript levels of E6 species were altered.
- EGF enrich medium saids in higher yield of HPV genome copies however; it does not have significant effect on morphology of keratinocytes and cell proliferation.
- Moreover, the basal cell environment enriched by the wound healing factors may play a vital role in the formation of a successful HPV infection. Therefore role of other growth factors should also be evaluated.

7. Future prospects

The work presented here is a contribution in human papillomavirus research in Pakistan and it also provides road map for future studies.

- Present study emphasizes on establishment of early diagnostic camps and public health education programs to hold time to time on national level to upgrade awareness and guidance to save the lives of females in Pakistan. Introduction of counselling programs to overcome the blockade of socio-cultural norms of the society.
- Inclusion of multiple sentinel sites i.e. comparison of rural and urban areas and collection of epidemiological data on genotype prevalence from throughout country will aid in estimating HPV burden in Pakistan and establishment of vaccination programs.
- Genotyping should not be limited to HPV 16 further HPV types should also be included in routine investigation for cervical cancer.
- Nucleotide sequencing should be incorporated in routine laboratory procedures for better diagnosis and therapeutics of cervical cancer.
- Frequently isolated new strains could be regarded as risks, there monitoring and identifying the route of transmission and potential risk factors should be addressed to minimize the spread.
- Studies on human papilloma virus genotypes in males should also be done.
- One of important future direction endorsed by this work is regarding HPV *in vitro* model system to study wound healing events. A complex mechanism controls the cell proliferation in NIKS-HPV16 cells and further analyses are necessary to elucidate the molecular pathways involved.
- Moreover, extensive *in vitro* model studies should be done in near future to understand the role of HPV infection in lesion formation.

8. References

Abdullah A, Qasim M, Shafiq M, Ijaz M, Parveen S, Murtaza S, Javed Q, Malik SA, Tarar SH, Mehmood S. 2016. Molecular diagnosis and phylogenetic analysis of human papillomavirus type-16 from suspected patients in Pakistan. Infectious agents and cancer 11(1), 1.

Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. 2001. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. The Journal of Immunology 166(12), 7556-7562.

Abreu AL, Souza RP, Gimenes F, Consolaro ME. 2012. A review of methods for detect human Papillomavirus infection. Virology journal 9(1), 262.

Adekunle OO. 2012. Cervical Intraepithelial Neoplasia (CIN)(Squamous Dysplasia). Intraepithelial Neoplasia, IntechOpen.

Akinola O, Fabamwo A, Oshodi Y, Banjo A, Odusanya O, Gbadegesin A, Tayo A. 2007. Efficacy of visual inspection of the cervix using acetic acid in cervical cancer screening: a comparison with cervical cytology. Journal of Obstetrics and Gynaecology 27(7), 703-705.

Al-Shaikh GK, Almussaed EM, Fayed AA, Khan FH, Syed SB, Al-Tamimi TN, Elmorshedy HN. 2014. Knowledge of Saudi female university students regarding cervical cancer and acceptance of the human papilloma virus vaccine. Saudi Medical Journal 35(10), 1223.

Albert S, Oguntayo O, Samaila M. 2012. Comparative study of visual inspection of the cervix using acetic acid (VIA) and Papanicolaou (Pap) smears for cervical cancer screening. Ecancermedicalscience 6, 262.

Aldohaian AI, Alshammari SA, Arafah DM. 2019a. Using the health belief model to assess beliefs and behaviors regarding cervical cancer screening among Saudi women: a cross-sectional observational study. BMC women's health 19(1), 6.

Aldohaian AI, Alshammari SA, Arafah DM. 2019b. Using the health belief model to assess beliefs and behaviors regarding cervical cancer screening among Saudi women: a cross-sectional observational study. Biomed Central Women's Health 19(1), 6.

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 amongst interns and nursing staff in Tertiary Care Hospitals in Karachi, Pakistan. PloS One 5(6), 11059.

Alsbeih G, Ahmed R, Al-Harbi N, Venturina LA, Tulbah A, Balaraj K. 2011. Prevalence and genotypes' distribution of human papillomavirus in invasive cervical cancer in Saudi Arabia. Gynecologic oncology 121(3), 522-526.

Anic GM, Lee JH, Stockwell H, Rollison DE, Wu Y, Papenfuss MR, Villa LL, Lazcano-Ponce E, Gage C, Silva RJC. 2011. Incidence and human papillomavirus (HPV) type distribution of genital warts in a multinational cohort of men: the HPV in men study. The Journal of infectious diseases 204(12), 1886-1892.

Ansari M, Mehdi G, Arif S, Ansari H, Khan T. 2012. Smear patterns and spectrum of premalignant and malignant cervical epithelial lesions in postmenopausal Indian women: A hospital-based study. Diagnostic cytopathology 40(11), 976-983.

Arbyn M, Tommasino M, Depuydt C, Dillner J. 2014. Are 20 human papillomavirus types causing cervical cancer? The Journal of pathology 234(4), 431-435.

Arroyo LS, Smelov V, Bzhalava D, Eklund C, Hultin E, Dillner J. 2013. Next generation sequencing for human papillomavirus genotyping. Journal of Clinical Virology 58(2), 437-442.

Arun R, Singh JP, Gupta SB. 2018. Cross-sectional study on visual inspection with acetic acid and pap smear positivity rates according to sociodemographic factors among rural married women of bareilly (Uttar Pradesh). Indian Journal of Community Medicine: official publication of Indian Association of Preventive and Social Medicine 43(2), 86.

Ault KA. 2006. Epidemiology and natural history of human papillomavirus infections in the female genital tract. Infectious diseases in obstetrics and gynecology 2006.

Autier P, Coibion M, Huet F, Grivegnee A-R. 1996. Transformation zone location and intraepithelial neoplasia of the cervix uteri. British journal of cancer 74(3), 488.

Aziz H, Iqbal H, Mahmood H, Fatima S, Faheem M, Sattar AA, Tabassum S, Napper S, Batool S, Rasheed N. 2018. Human papillomavirus infection in females with normal cervical cytology: Genotyping and phylogenetic analysis among women in Punjab, Pakistan. International Journal of Infectious Diseases 66, 83-89.

Baay M, Quint W, Koudstaal J, Hollema H, Duk JM, Burger M, Stolz E, Herbrink P. 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 34(3), 745-747.

Bacalbasa N, Balescu I, Suciu I, Dima S, Suciu N. 2018. HPV Infection and Vulvar Cancer. Current Perspectives in Human Papillomavirus, IntechOpen.

Balachandran I. 2012. Human papillomavirus and pap smear: A review. American Journal of Lifestyle Medicine 6(1), 31-44.

Basu P, Mittal S, Vale DB, Kharaji YC. 2018. Secondary prevention of cervical cancer. Best Practice and Research Clinical Obstetrics and Gynaecology 47, 73-85.

Bayo S, Bosch FX, de Sanjosé S, Muñoz N, Combita AL, Coursaget P, Diaz M, Dolo A, van den Brule AJ, Meijer CJ. 2002. Risk factors of invasive cervical cancer in Mali. International journal of epidemiology 31(1), 202-209.

Bedoya AM, Jaramillo R, Baena A, Castaño J, Olaya N, Zea AH, Herrero R, Sanchez GI. 2013. Location and density of immune cells in precursor lesions and cervical cancer. Cancer Microenvironment 6(1), 69-77.

Bergvall M, Gagnon D, Titolo S, Lehoux M, D'Abramo CM, Melendy T, Archambault J. 2016. Requirement for the E1 helicase C-terminal domain in papillomavirus DNA replication in vivo. Journal of virology 90(6), 3198-3211.

Bernard H-U. 2013. Regulatory elements in the viral genome. Virology 445(1-2), 197-204.

Bernard H-U, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers E-M. 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 401(1), 70-79.

Bernard H-U, Chan S-Y, Manos MM, Ong C-K, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. 1994. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. Journal of Infectious Diseases 170(5), 1077-1085.

Bertelsen BI, Kugarajh K, Skar R, Laerum OD. 2006. HPV subtypes in cervical cancer biopsies between 1930and 2004: detection using general primer pair PCR and sequencing. Virchows Archiv 449(2), 141-147.

Bhatla N, Aoki D, Sharma DN, Sankaranarayanan R. 2018. Cancer of the cervix uteri. International Journal of Gynecology & Obstetrics 143, 22-36.

Bhurgri Y, Nazir K, Shaheen Y, Usman A, Faridi N, Bhurgri H, Malik J, Bashir I, Bhurgri A, Kayani N. 2007. Patho-epidemiology of cancer cervix in Karachi South. Asian Pacific Journal of Cancer Prevention 8(3), 357.

Blaustein M, Pelisch F, Srebrow A. 2007. Signals, pathways and splicing regulation. The international journal of biochemistry & cell biology 39(11), 2031-2048.

Blomberg M, Friis S, Munk C, Bautz A, Kjaer SK. 2012. Genital warts and risk of cancer: a Danish study of nearly 50 000 patients with genital warts. The Journal of infectious diseases 205(10), 1544-1553.

Boda D, Docea AO, Calina D, Ilie MA, Caruntu C, Zurac S, Neagu M, Constantin C, Branisteanu DE, Voiculescu V. 2018. Human papilloma virus: Apprehending the link with carcinogenesis and unveiling new research avenues. International journal of oncology 52(3), 637-655.

Bodily J, Laimins LA. 2011. Persistence of human papillomavirus infection: keys to malignant progression. Trends in microbiology 19(1), 33-39.

Bodnar RJ. 2013. Epidermal growth factor and epidermal growth factor receptor: the Yin and Yang in the treatment of cutaneous wounds and cancer. Advances in wound care 2(1), 24-29.

Bohm S, Wilczynski S, Pfister H, Iftner T. 1993. The predominant mRNA class in HPV16-infected genital neoplasias does not encode the e6 or the E7 protein. International journal of cancer 55(5), 791-798.

Bomfim S, Santana-Franco E, Bahamondes L. 2005. Visual inspection with acetic acid for cervical cancer detection. International Journal of Gynecology and Obstetrics 88(1), 65-66.

Bora K, Chowdhury M, Mahanta LB, Kundu MK, Das AK. 2017. Automated classification of Pap smear images to detect cervical dysplasia. Computer Methods and Programs in Biomedicine 138, 31-47.

Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. The EMBO journal 3(5), 1151-1157.

Bősze P. 2013. The first vaccine against cancer: the human papillomavirus vaccine. Orvosi hetilap 154(16), 603-618.

Boulet GA, Micalessi IM, Horvath CA, Benoy IH, Depuydt CE, Bogers JJ. 2010. Nucleic acid sequence-based amplification assay for human papillomavirus mRNA detection and typing: evidence for DNA amplification. Journal of clinical microbiology 48(7), 2524-2529.

Bousarghin L, Touzé A, Sizaret P-Y, Coursaget P. 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. Journal of virology 77(6), 3846-3850.

Bravo IG, Félez-Sánchez M. 2015. PapillomavirusesViral evolution, cancer and evolutionary medicine. Evolution, medicine, and public health 2015(1), 32-51.

Bravo IG, de Sanjosé S, Gottschling M. 2010. The clinical importance of understanding the evolution of papillomaviruses. Trends in microbiology 18(10), 432-438.

Brightwell JR, Riddle SL, Eiferman RA, Valenzuela P, Barr PJ, Merryweather JP, Schultz GS. 1985. Biosynthetic human EGF accelerates healing of Neodecadron-treated primate corneas. Invest Ophthalmol Vis Sci 26(1), 105-110.

Briolat J, Dalstein V, Saunier M, Joseph K, Caudroy S, Prétet JL, Birembaut P, Clavel C. 2007. HPV prevalence, viral load and physical state of HPV-16 in cervical smears of patients with different grades of CIN. International journal of cancer 121(10), 2198-2204.

Broder S. 1992. Rapid communication—the Bethesda system for reporting cervical/vaginal cytologic diagnoses—report of the 1991 Bethesda workshop. JAMA 267(14), 1892-1892.

Bruni L, Diaz M, Castellsagué M, Ferrer E, Bosch FX, de Sanjosé S. 2010. Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings. Journal of Infectious Diseases 202(12), 1789-1799.

Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, Brotons M, Mena M, Cosano R, Muñoz J. 2015. Human papillomavirus and related diseases in the world. Summary report 20140822.

Buck CB, Day PM, Trus BL. 2013. The papillomavirus major capsid protein L1. Virology 445(1-2), 169-174.

Bukhari MH, Saba K, Qamar S, Majeed MM, Niazi S, Naeem S. 2012. Clinicopathological importance of Papanicolaou smears for the diagnosis of premalignant and malignant lesions of the cervix. Journal of Cytology/Indian Academy of Cytologists 29(1), 20.

Burd EM. 2003. Human papillomavirus and cervical cancer. Clinical microbiology reviews 16(1), 1-17.

Burd EM. 2016. Human papillomavirus laboratory testing: the changing paradigm. Clinical microbiology reviews 29(2), 291-319.

Cancer ICoESoC. 2007. Cervical cancer and hormonal contraceptives: collaborative reanalysis of individual data for 16 573 women with cervical cancer and 35 509 women without cervical cancer from 24 epidemiological studies. The Lancet 370(9599), 1609-1621.

Carozzi F, Gillio-Tos A, Confortini M, Del Mistro A, Sani C, De Marco L, Girlando S, Rosso S, Naldoni C, Dalla Palma P. 2013. Risk of high-grade cervical intraepithelial neoplasia during follow-up in HPV-positive women according to baseline p16-INK4A results: a prospective analysis of a nested substudy of the NTCC randomised controlled trial. The lancet oncology 14(2), 168-176.

Carson A, Khan SA. 2006. Characterization of transcription factor binding to human papillomavirus type 16 DNA during cellular differentiation. Journal of virology 80(9), 4356-4362.

Carter JJ, Madeleine MM, Shera K, Schwartz SM, Cushing-Haugen KL, Wipf GC, Porter P, Daling JR, McDougall JK, Galloway DA. 2001. Human papillomavirus 16 and 18 L1 serology compared across anogenital cancer sites. Cancer research 61(5), 1934-1940.

Carvalho MOO, Almeida RW, Leite F, Fellows IB, Teixeira MH, Oliveira LH, Cavalcanti S. 2003. Detection of human papillomavirus DNA by the hybrid capture assay. Brazilian Journal of Infectious Diseases 7(2), 121-125.

Carvalho NdO, del Castillo DM, Perone C, Januário JN, Melo VHd, Brasileiro Filho G. 2010. Comparison of HPV genotyping by type-specific PCR and sequencing. Memórias do Instituto Oswaldo Cruz 105(1), 73-78.

Cason J, Mant CA. 2005. High-risk mucosal human papillomavirus infections during infancy & childhood. Journal of Clinical Virology 32, 52-58.

Castellsagué X, Bosch FX, Muñoz N. 2003. The male role in cervical cancer. salud pública de méxico 45, 345-353.

Castillo A. 2013. Human papillomavirus and carcinogenesis in the upper aero-digestive tract. Carcinogenesis, 45.

Castle PE, Murokora D, Perez C, Alvarez M, Quek SC, Campbell C. 2017. Treatment of cervical intraepithelial lesions. International Journal of Gynecology & Obstetrics 138, 20-25.

Cetisli NE, Top ED, Işık G. 2016. Cervical Cancer and Pap Smear Test Health Beliefs and Health-Promoting Lifestyle of Women in Turkey. International Journal of Caring Sciences 9(2), 630-639.

Chen Z-x, Riggs AD. 2011. DNA methylation and demethylation in mammals. Journal of Biological Chemistry 286(21), 18347-18353.

Chesney J, Bucala R. 2000. Peripheral blood fibrocytes: mesenchymal precursor cells and the pathogenesis of fibrosis. Current rheumatology reports 2(6), 501-505.

Cho C-Y, Lo Y-C, Hung M-C, Lai C-C, Chen C-J, Wu K-G. 2017. Risk of cancer in patients with genital warts: A nationwide, population-based cohort study in Taiwan. PloS one 12(8), e0183183.

Chong T, Apt D, Gloss B, Isa M, Bernard H-U. 1991. The enhancer of human papillomavirus type 16: binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription. Journal of Virology 65(11), 5933-5943.

Clark RA, Ashcroft GS, Spencer MJ, Larjava H, Ferguson MW. 1996. Re-epithelialization of normal human excisional wounds is associated with a switch from alpha v beta 5 to alpha v beta 6 integrins. Br J Dermatol 135(1), 46-51.

Cogliano V, Grosse Y, Baan R, Straif K, Secretan B, El Ghissassi F. 2005. Carcinogenicity of combined oestrogen-progestagen contraceptives and menopausal treatment. The lancet oncology 6(8), 552-553.

Cohen S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. Journal of Biological Chemistry 237(5), 1555-1562.

Collins AS, Nakahara T, Do A, Lambert PF. 2005. Interactions with pocket proteins contribute to the role of human papillomavirus type 16 E7 in the papillomavirus life cycle. J Virol 79(23), 14769-14780.

Cribbs RK, Harding PA, Luquette MH, Besner GE. 2002. Endogenous production of heparin-binding EGF-like growth factor during murine partial-thickness burn wound healing. The Journal of burn care & rehabilitation 23(2), 116-125.

Cubie HA. 2013. Diseases associated with human papillomavirus infection. Virology 445(1-2), 21-34.

Daling JR, Madeleine MM, Schwartz SM, Shera KA, Carter JJ, McKnight B, Porter PL, Galloway DA, McDougall JK, Tamimi H. 2002. A population-based study of squamous cell vaginal cancer: HPV and cofactors. Gynecologic oncology 84(2), 263-270.

Daling JR, Madeleine MM, Johnson LG, Schwartz SM, Shera KA, Wurscher MA, Carter JJ, Porter PL, Galloway DA, McDougall JK. 2005. Penile cancer: importance of circumcision, human papillomavirus and smoking in in situ and invasive disease. International journal of cancer 116(4), 606-616.

Daniyal M, Akhtar N, Ahmad S, Fatima U, Akram M, Asif HM. 2015. Update knowledge on cervical cancer incidence and prevalence in Asia. Asian Pacific Journal of Cancer Prevention 16(9), 3617-3620.

Das CM, Shah N, Ghori A, Khursheed F, Zaheen Z. 2013. Prevalence and risk factors for cervical intraepithelial neoplasia in patients attending gynecological outpatient department of tertiary care hospital. Journal of Liaquat University of Medical and Health Sciences Jamshoro, Pakistan, 12(01), 44.

David M-P, Van Herck K, Hardt K, Tibaldi F, Dubin G, Descamps D, Van Damme P. 2009. Long-term persistence of anti-HPV-16 and-18 antibodies induced by vaccination with the AS04-adjuvanted cervical cancer vaccine: modeling of sustained antibody responses. Gynecologic oncology 115(3), S1-S6.

Davy CE, Jackson DJ, Raj K, Peh WL, Southern SA, Das P, Sorathia R, Laskey P, Middleton K, Nakahara T. 2005. Human papillomavirus type 16 E1∧ E4-induced G2 arrest is associated with cytoplasmic retention of active Cdk1/cyclin B1 complexes. Journal of virology 79(7), 3998-4011.

de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE, Lloveras B, Tous S, Felix A, Bravo LE, Shin H-R. 2010. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. The lancet oncology 11(11), 1048-1056.

de Villiers E-M. 2013. Cross-roads in the classification of papillomaviruses. Virology 445(1-2), 2-10.

De Villiers E-M, Fauquet C, Broker TR, Bernard H-U, zur Hausen H. 2004. Classification of papillomaviruses. Virology 324(1), 17-27.

Del Río-Ospina L, Soto-De León SC, Camargo M, Sánchez R, Mancilla CL, Patarroyo ME, Patarroyo MA. 2016. The prevalence of high-risk HPV types and factors determining infection in female colombian adolescents. PloS one 11(11), e0166502.

Deligeoroglou E, Giannouli A, Athanasopoulos N, Karountzos V, Vatopoulou A, Dimopoulos K, Creatsas G. 2013. HPV infection: immunological aspects and their utility in future therapy. Infectious diseases in obstetrics and gynecology 2013.

Denny L, Quinn M, Sankaranarayanan R. 2006. Screening for cervical cancer in developing countries. Vaccine 24, 71-77.

Diaz ML. 2008. Human papilloma virus–prevention and treatment. Obstetrics and gynecology clinics of North America 35(2), 199-217.

Doorbar J. 2005. The papillomavirus life cycle. Journal of clinical virology 32, 7-15.

Doorbar J. 2006. Molecular biology of human papillomavirus infection and cervical cancer. Clinical science 110(5), 525-541.

Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. 2015. Human papillomavirus molecular biology and disease association. Reviews in medical virology 25, 2-23.

Doorbar J, Quint W, Banks L, Bravo IG, Stoler M, Broker TR, Stanley MA. 2012. The biology and life-cycle of human papillomaviruses. Vaccine 30, F55-F70.

Doorn L-Jv, Kleter B, Quint WG. 2001. Molecular detection and genotyping of human papillomavirus. Expert review of molecular diagnostics 1(4), 394-402.

Du P-L, Wu K-S, Fang J-Y, Zeng Y, Xu Z-X, Tang W-R, Xu X-L, Lin K. 2015. Cervical cancer mortality trends in China, 1991-2013, and predictions for the future. Asian Pacific Journal of Cancer Prevention 16(15), 6391-6396.

Dunlop S, Coyte PC, McIsaac W. 2000. Socio-economic status and the utilisation of physicians' services: results from the Canadian National Population Health Survey. Social science & medicine 51(1), 123-133.

Dürst M, Gissmann L, Ikenberg H, Zur Hausen H. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proceedings of the National Academy of Sciences 80(12), 3812-3815.

Egawa N, Egawa K, Griffin H, Doorbar J. 2015. Human papillomaviruses; epithelial tropisms, and the development of neoplasia. Viruses 7(7), 3863-3890.

Egawa N, Nakahara T, Ohno S-i, Narisawa-Saito M, Yugawa T, Fujita M, Yamato K, Natori Y, Kiyono T. 2012. The E1 protein of human papillomavirus type 16 is dispensable for maintenance replication of the viral genome. Journal of virology 86(6), 3276-3283.

Egeblad M, Nakasone ES, Werb Z. 2010. Tumors as organs: complex tissues that interface with the entire organism. Developmental cell 18(6), 884-901.

Eklund C, Zhou T, Dillner J. 2010. Global proficiency study of human papillomavirus genotyping. Journal of clinical microbiology 48(11), 4147-4155.

Elson DA, Riley RR, Lacey A, Thordarson G, Talamantes FJ, Arbeit JM. 2000. Sensitivity of the cervical transformation zone to estrogen-induced squamous carcinogenesis. Cancer Research 60(5), 1267-1275.

Eming SA, Brachvogel B, Odorisio T, Koch M. 2007. Regulation of angiogenesis: wound healing as a model. Progress in histochemistry and cytochemistry 42(3), 115-170.

Erickson BK, Landers EE, Huh WK. 2014. Update on vaccination clinical trials for HPV-related disease. Clinical therapeutics 36(1), 8-16.

Ermel A, Qadadri B, Morishita A, Miyagawa I, Yamazaki G, Weaver B, Tu W, Tong Y, Randolph M, Cramer H. 2010. Human papillomavirus detection and typing in thin prep cervical cytologic specimens comparing the Digene Hybrid Capture II Assay, the Roche Linear Array HPV Genotyping Assay, and the Kurabo GeneSquare Microarray Assay. Journal of virological methods 169(1), 154-161.

Fan Y, Sanyal S, Bruzzone R. 2018. Breaking bad: how viruses subvert the cell cycle. Frontiers in cellular and infection microbiology 8.

Fehrmann F, Klumpp DJ, Laimins LA. 2003. Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. Journal of virology 77(5), 2819-2831.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin D, Forman D, Bray F. 2014. GLOBOCAN 2012 v1. 0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. 2013; Lyon, France: International Agency for Research on Cancer. globocan. iarc. fr/Default. aspx.

Finnen RL, Erickson KD, Chen XS, Garcea RL. 2003. Interactions between papillomavirus L1 and L2 capsid proteins. Journal of virology 77(8), 4818-4826.

Finocchario-Kessler S, Wexler C, Maloba M, Mabachi N, Ndikum-Moffor F, Bukusi E. 2016. Cervical cancer prevention and treatment research in Africa: a systematic review from a public health perspective. Biomed Central Women's Health 16(1), 29.

Firth AE, Brierley I. 2012. Non-canonical translation in RNA viruses. The Journal of general virology 93(Pt 7), 1385.

Fontecha N, Nieto MC, Andía D, Cisterna R, Basaras M. 2017. RNA extraction method is crucial for human papillomavirus E6/E7 oncogenes detection. Virology journal 14(1), 50.

Foresta C, Patassini C, Bertoldo A, Menegazzo M, Francavilla F, Barzon L, Ferlin A. 2011. Mechanism of human papillomavirus binding to human spermatozoa and fertilizing ability of infected spermatozoa. PloS one 6(3), e15036.

Forman D, de Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L, Vignat J, Ferlay J, Bray F, Plummer M. 2012. Global burden of human papillomavirus and related diseases. Vaccine 30, F12-F23.

Franceschi S, Herrero R, Clifford GM, Snijders PJ, Arslan A, Anh PTH, Bosch FX, Ferreccio C, Hieu NT, Lazcano-Ponce E. 2006. Variations in the age-specific curves of human papillomavirus prevalence in women worldwide. International journal of cancer 119(11), 2677-2684.

Frazer IH, Leggatt GR, Mattarollo SR. 2011. Prevention and treatment of papillomavirus-related cancers through immunization. Annual review of immunology 29, 111-138.

Fuchs E, Tumbar T, Guasch G. 2004. Socializing with the neighbors: stem cells and their niche. Cell 116(6), 769-778.

Gami B, Kubba F, Ziprin P. 2014. Human papilloma virus and squamous cell carcinoma of the anus. Clinical Medicine Insights: Oncology 8, CMO. S13241.

Garibyan L, Avashia N. 2013. Research techniques made simple: polymerase chain reaction (PCR). The Journal of investigative dermatology 133(3), e6.

Garland SM, Bhatla N, Ngan HY. 2012. Cervical cancer burden and prevention strategies: Asia Oceania perspective. Cancer Epidemiology and Prevention Biomarkers 21(9), 1414-1422.

Garland SM, Steben M, Sings HL, James M, Lu S, Railkar R, Barr E, Haupt RM, Joura EA. 2009. Natural history of genital warts: analysis of the placebo arm of 2 randomized phase III trials of a quadrivalent human papillomavirus (types 6, 11, 16, and 18) vaccine. The Journal of infectious diseases 199(6), 805-814.

Gasparini R, Panatto D. 2009. Cervical cancer: from Hippocrates through Rigoni-Stern to zur Hausen.

Genther SM, Sterling S, Duensing S, Münger K, Sattler C, Lambert PF. 2003. Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle. Journal of virology 77(5), 2832-2842.

Gilbert MTP, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, Van Marck E, Worobey M. 2007. The isolation of nucleic acids from fixed, paraffin-embedded tissues–which methods are useful when? PLoS One 2(6), e537.

Giles M, Garland S. 2006. A study of women's knowledge regarding human papillomavirus infection, cervical cancer and human papillomavirus vaccines. Australian and New Zealand Journal of Obstetrics and Gynaecology 46(4), 311-315.

Gok M, Heideman DA, van Kemenade FJ, Berkhof J, Rozendaal L, Spruyt JW, Voorhorst F, Beliën JA, Babović M, Snijders PJ. 2010. HPV testing on self collected cervicovaginal lavage specimens as screening method for women who do not attend cervical screening: cohort study. British Medical Journal 340, 1040.

Goldie S, Kim J, Kobus K, Holtan M, Kuntz K, Salomon J. 2006. Cost-effectiveness analysis of prophylactic human papillomavirus vaccination and screening in Brazil. Poster presentation. Pages 1-7. 23rd International Papillomavirus Conference and Clinical Workshop.

Grassmann K, Rapp B, Maschek H, Petry KU, Iftner T. 1996. Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new cell line containing high copy numbers of episomal HPV-16 DNA. J Virol 70(4), 2339-2349.

Gravitt P, Peyton C, Apple R, Wheeler C. 1998. Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol 36(10), 3020-3027.

Gravitt P, Peyton C, Alessi T, Wheeler C, Coutlee F, Hildesheim A, Schiffman M, Scott D, Apple R. 2000. Improved amplification of genital human papillomaviruses. Journal of clinical microbiology 38(1), 357-361.

Gravitt PE, Paul P, Katki HA, Vendantham H, Ramakrishna G, Sudula M, Kalpana B, Ronnett BM, Vijayaraghavan K, Shah KV. 2010. Effectiveness of VIA, Pap, and HPV DNA testing in a cervical cancer screening program in a peri-urban community in Andhra Pradesh, India. PloS One 5(10), 13711.

Gray W, Kocjan G. 2010. Diagnostic cytopathology: Elsevier Health Sciences.

Graziottin A, Serafini A. 2009. HPV infection in women: psychosexual impact of genital warts and intraepithelial lesions. The journal of sexual medicine 6(3), 633-645.

Grossman S. 2013. Porth's pathophysiology: Concepts of altered health states: Lippincott Williams & Wilkins.

Gul S, Murad S, Javed A. 2015. Prevalence of High risk Human Papillomavirus in cervical dysplasia and cancer samples from twin cities in Pakistan. International Journal of Infectious Diseases 34, 14-19.

Haase I, Evans R, Pofahl R, Watt FM. 2003. Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1-and EGF-dependent signalling pathways. Journal of cell science 116(15), 3227-3238.

Haedicke J, Iftner T. 2016. A review of the clinical performance of the Aptima HPV assay. Journal of Clinical Virology 76, S40-S48.

Haider G, Parveen Z, Anjum F, Munir A. 2013. Pap smear, an important screening tool to detect precancerous stage of carcinoma of cervix. Journal of Ayub Medical College Abbottabad 25(1-2), 26-27.

Harper DM, DeMars LR. 2017. HPV vaccines–a review of the first decade. Gynecologic oncology 146(1), 196-204.

Hastert TA, Beresford SA, Sheppard L, White E. 2015. Disparities in cancer incidence and mortality by area-level socioeconomic status: a multilevel analysis. J Epidemiol Community Health 69(2), 168-176.

Haws ALF, He Q, Rady PL, Zhang L, Grady J, Hughes TK, Stisser K, Konig R, Tyring SK. 2004. Nested PCR with the PGMY09/11 and GP5+/6+ primer sets improves detection of HPV DNA in cervical samples. J Virol Methods 122(1), 87-93.

Heather JM, Chain B. 2016. The sequence of sequencers: The history of sequencing DNA. Genomics 107(1), 1-8.

Hejmadi M. 2009. Introduction to cancer biology: Bookboon.

Heldin C-H, Westermark B. 1999. Mechanism of action and in vivo role of platelet-derived growth factor. Physiological reviews 79(4), 1283-1316.

Herfs M, Yamamoto Y, Laury A, Wang X, Nucci MR, McLaughlin-Drubin ME, Münger K, Feldman S, McKeon FD, Xian W. 2012. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. Proceedings of the National Academy of Sciences 109(26), 10516-10521.

Hildesheim A, Herrero R, Castle PE, Wacholder S, Bratti M, Sherman ME, Lorincz A, Burk RD, Morales J, Rodríguez AC. 2001. HPV co-factors related to the development of cervical cancer: results from a population-based study in Costa Rica. British journal of cancer 84(9), 1219.

Ho L, Chan SY, Burk RD, Das B, Fujinaga K, Icenogle JP, Kahn T, Kiviat N, Lancaster W, Mavromara-Nazos P. 1993. The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. Journal of virology 67(11), 6413-6423.

Hoda RS, Loukeris K, Abdul-Karim FW. 2013. Gynecologic cytology on conventional and liquid-based preparations: A comprehensive review of similarities and differences. Diagnostic cytopathology 41(3), 257-278.

Hoffman SR, Le T, Lockhart A, Sanusi A, Dal Santo L, Davis M, McKinney DA, Brown M, Poole C, Willame C. 2017. Patterns of persistent HPV infection after treatment for cervical intraepithelial neoplasia (CIN): A systematic review. International journal of cancer 141(1), 8-23.

Hoory T, Monie A, Gravitt P, Wu T-C. 2008. Molecular epidemiology of human papillomavirus. Journal of the Formosan Medical Association 107(3), 198-217.

Howie HL, Katzenellenbogen RA, Galloway DA. 2009. Papillomavirus E6 proteins. Virology 384(2), 324-334.

Hu J, Budgeon LR, Cladel NM, Culp TD, Balogh KK, Christensen ND. 2007. Detection of L1, infectious virions and anti-L1 antibody in domestic rabbits infected with cottontail rabbit papillomavirus. Journal of General Virology 88(12), 3286-3293.

Hubbard RA. 2003. Human papillomavirus testing methods. Archives of pathology & laboratory medicine 127(8), 940-945.

hui Xu H, Lin A, hong Chen Y, shan Dong S, wu Shi W, zheng Yu J, hua Yan W. 2017. Prevalence characteristics of cervical human papillomavirus (HPV) genotypes in the Taizhou area, China: a cross-sectional study of 37 967 women from the general population. BMJ open 7(6), e014135.

Husain N, Neyaz A. 2017. Human papillomavirus associated head and neck squamous cell carcinoma: controversies and new concepts. Journal of oral biology and craniofacial research 7(3), 198-205.

Iaconis L, Hyjek E, Ellenson LH, Pirog EC. 2007. p16 and Ki-67 immunostaining in atypical immature squamous metaplasia of the uterine cervix: correlation with human papillomavirus detection. Archives of pathology & laboratory medicine 131(9), 1343-1349.

Iruela-Arispe LM, Dvorak HF. 1997. Angiogenesis: a dynamic balance of stimulators and inhibitors. Thrombosis and haemostasis 78(01), 672-677.

Ishikawa M, Fujii T, Saito M, Nindl I, Ono A, Kubushiro K, Tsukazaki K, Mukai M, Nozawa S. 2006. Overexpression of p16INK4a as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. International Journal of Gynecologic Cancer 16(1), 347-353.

Javier RT, Butel JS. 2008. The history of tumor virology. Cancer research 68(19), 7693-7706.

Jbeen M, Gul F, Javed N, Mehroz S. 2013. Frequency of cervical intraepithelial neoplasia in women attending Liaqat Memorial Hospital Kohat. Khyber Medical University Journal 5, 132-136.

Jensen K, Schmiedel S, Norrild B, Frederiksen K, Iftner T, Kjaer S. 2013. Parity as a cofactor for high-grade cervical disease among women with persistent human papillomavirus infection: a 13-year follow-up. British journal of cancer 108(1), 234.

Jia R, Liu X, Tao M, Kruhlak M, Guo M, Meyers C, Baker CC, Zheng ZM. 2009. Control of the papillomavirus early-to-late switch by differentially expressed SRp20. J Virol 83(1), 167-180.

Jing Y, Wang T, Chen Z, Ding X, Xu J, Mu X, Cao M, Chen H. 2018. Phylogeny and polymorphism in the long control regions E6, E7, and L1 of HPV Type 56 in women from southwest China. Molecular medicine reports 17(5), 7131-7141.

Johansson C, Schwartz S. 2013. Regulation of human papillomavirus gene expression by splicing and polyadenylation. Nature reviews Microbiology 11(4), 239.

Johansson C, Somberg M, Li X, Winquist EB, Fay J, Ryan F, Pim D, Banks L, Schwartz S. 2012. HPV-16 E2 contributes to induction of HPV-16 late gene expression by inhibiting early polyadenylation. The EMBO journal 31(14), 3212-3227.

Joyce JG, Tung J-S, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU, Keller PM. 1999. The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. Journal of Biological Chemistry 274(9), 5810-5822.

Kadaja M, Silla T, Ustav E, Ustav M. 2009. Papillomavirus DNA replication—from initiation to genomic instability. Virology 384(2), 360-368.

Kadam MV, Doke P, Mathews M. 2018. Effect of planned teaching on the knowledge,attitudes and practices of women pertaining to cervical cancer,screening and its secondary prevention. Indian Journal of Applied Research 8(2), 46-49.

Kajitani N, Satsuka A, Kawate A, Sakai H. 2012. Productive lifecycle of human papillomaviruses that depends upon squamous epithelial differentiation. Frontiers in microbiology 3, 152.

Kang YJ, Smith M, Barlow E, Coffey K, Hacker N, Canfell K. 2017. Vulvar cancer in high-income countries: Increasing burden of disease. International journal of cancer 141(11), 2174-2186.

Kanodia S, Fahey LM, Kast WM. 2007. Mechanisms used by human papillomaviruses to escape the host immune response. Current cancer drug targets 7(1), 79-89.

Kariche N, Hortal MT, Benyahia S, Alemany L, Moulaï N, Clavero O, Muñoz M, Ouahioune W, Djennaoui D, Touil-Boukoffa C. 2018. Comparative assessment of HPV, alcohol and tobacco etiological fractions in Algerian patients with laryngeal squamous cell carcinoma. Infectious agents and cancer 13(1), 8.

Kchouk M, Gibrat J-F, Elloumi M. 2017. Generations of sequencing technologies: From first to next generation. Biology and Medicine 9(3).

Kelesidis T, Aish L, Steller MA, Aish IS, Shen J, Foukas P, Panayiotides J, Petrikkos G, Karakitsos P, Tsiodras S. 2011. Human papillomavirus (HPV) detection using in situ hybridization in histologic samples: correlations with cytologic changes and polymerase chain reaction HPV detection. American journal of clinical pathology 136(1), 119-127.

Keys HM, Bundy BN, Stehman FB, Muderspach LI, Chafe WE, Suggs CL, Walker JL, Gersell D. 1999. Cisplatin, radiation, and adjuvant hysterectomy compared with radiation and adjuvant hysterectomy for bulky stage IB cervical carcinoma. New England Journal of Medicine 340(15), 1154-1161.

Khan I, Khanzada F, Avais A. 2013. Pap smear screening in women presenting with chronic discharge at a tertiary care setting. Annals of Pakistan Institute of Medical Sciences 9, 61-63.

Khan S, Jaffer NN, Khan MN, Rai MA, Shafiq M, Ali A, Pervez S, Khan N, Aziz A, Ali SH. 2007a. Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma. International Journal of Infectious Diseases 11, 313-317.

Khan S, Jaffer NN, Khan MN, Rai MA, Shafiq M, Ali A, Pervez S, Khan N, Aziz A, Ali SH. 2007b. Human papillomavirus subtype 16 is common in Pakistani women with cervical carcinoma. International journal of infectious diseases 11(4), 313-317.

Khattak ST, Khattak I, Naheed T, Akhtar S, Jamal T. 2006. Detection of abnormal cervical cytology by pap smears. Gomal Journal of Medical Sciences 4(2), 74-77.

Kidd LC, Chaing S, Chipollini J, Giuliano AR, Spiess PE, Sharma P. 2017. Relationship between human papillomavirus and penile cancer—implications for prevention and treatment. Translational Andrology and Urology 6(5), 791.

Kim J, Kim BK, Lee CH, Seo SS, Park S-Y, Roh J-W. 2012. Human papillomavirus genotypes and cofactors causing cervical intraepithelial neoplasia and cervical cancer in Korean women. International Journal of Gynecologic Cancer 22(9), 1570-1576.

Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. 2009. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proceedings of the National Academy of Sciences 106(48), 20458-20463.

Kjaer SK, Chackerian B, Van Den Brule AJ, Svare EI, Paull G, Walbomers JM, Schiller JT, Bock JE, Sherman ME, Lowy DR. 2001. High-risk human papillomavirus is sexually transmitted: evidence from a follow-up study of virgins starting sexual activity (intercourse). Cancer Epidemiology and Prevention Biomarkers 10(2), 101-106.

Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M. 2002. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. The American journal of surgical pathology 26(11), 1389-1399.

Kokka F, Bryant A, Brockbank E, Powell M, Oram D. 2015. Hysterectomy with radiotherapy or chemotherapy or both for women with locally advanced cervical cancer. Cochrane Database of Systematic Reviews (4).

Koliopoulos G, Arbyn M, Martin-Hirsch P, Kyrgiou M, Prendiville W, Paraskevaidis E. 2007. Diagnostic accuracy of human papillomavirus testing in primary cervical screening: a systematic review and meta-analysis of non-randomized studies. Gynecologic oncology 104(1), 232-246.

Kolly C, Suter MM, Müller EJ. 2005. Proliferation, cell cycle exit, and onset of terminal differentiation in cultured keratinocytes: pre-programmed pathways in control of C-Myc and Notch1 prevail over extracellular calcium signals. Journal of investigative dermatology 124(5), 1014-1025.

Kösel S, Burggraf S, Mommsen J, Engelhardt W, Olgemöller B. 2003. Type-specific detection of human papillomaviruses in a routine laboratory setting–improved sensitivity and specificity of PCR and sequence analysis compared to direct hybridisation. Clinical chemistry and laboratory medicine 41(6), 787-791.

Kozak M. 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. The Journal of cell biology 115(4), 887-903.

Kreimer AR, Clifford GM, Boyle P, Franceschi S. 2005. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiology and Prevention Biomarkers 14(2), 467-475.

LaCour DE, Trimble C. 2012. Human papillomavirus in infants: transmission, prevalence, and persistence. Journal of pediatric and adolescent gynecology 25(2), 93-97.

Lam JUH, Rebolj M, Dugué P-A, Bonde J, von Euler-Chelpin M, Lynge E. 2014. Condom use in prevention of Human Papillomavirus infections and cervical neoplasia: systematic review of longitudinal studies. Journal of medical screening 21(1), 38-50.

Laurson J, Khan S, Chung R, Cross K, Raj K. 2010. Epigenetic repression of E-cadherin by human papillomavirus 16 E7 protein. Carcinogenesis 31(5), 918-926.

Lee LG, Connell CR, Woo SL, Cheng RD, McArdle BF, Fuller CW, Halloran ND, Wilson RK. 1992. DNA sequencing with dye-labeled terminators and T7 DNA polymerase: effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. Nucleic acids research 20(10), 2471-2483.

Lee SH, Vigliotti JS, Vigliotti VS, Jones W. 2014. From human papillomavirus (HPV) detection to cervical cancer prevention in clinical practice. Cancers 6(4), 2072-2099.

Levenson S, Geever E, Crowley L, Oates III J, Berard C, Rosen H. 1965. Healing of rat skin wounds. Annals of surgery 161(2), 293.

Lindh M, Görander S, Andersson E, Horal P, Mattsby-Balzer I, Ryd W. 2007. Real-time Taqman PCR targeting 14 human papilloma virus types. Journal of Clinical Virology 40(4), 321-324.

Liu Z-C, Liu W-D, Liu Y-H, Ye X-H, Chen S-D. 2015. Multiple sexual partners as a potential independent risk factor for cervical cancer: a meta-analysis of epidemiological studies. Asian Pac J Cancer Prev 16(9), 3893-3900.

Lorenz TC. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. JoVE (Journal of Visualized Experiments) (63), e3998.

Louie K, De Sanjose S, Diaz M, Castellsague X, Herrero R, Meijer C, Shah K, Franceschi S, Munoz N, Bosch F. 2009. Early age at first sexual intercourse and early pregnancy are risk factors for cervical cancer in developing countries. British Journal of Cancer 100(7), 1191.

Loya A, Serrano B, Rasheed F, Tous S, Hassan M, Clavero O, Raza M, De Sanjosé S, Bosch FX, Alemany L. 2016. Human Papillomavirus Genotype Distribution in Invasive Cervical Cancer in Pakistan. Cancers 8(8), 72.

Luff R, Kurman R, Solomon D. 1992. The Bethesda System for reporting cervical and vaginal cytologic diagnoses: report of the 1991 Bethesda workshop. Journal of Family Practice 35(1), 98-101.

Macedo ACL, Gonçalves JCN, Bavaresco DV, Grande AJ, Chiaramonte Silva N, Rosa MI. 2019. Accuracy of mRNA HPV Tests for Triage of Precursor Lesions and Cervical Cancer: A Systematic Review and Meta-Analysis. Journal of Oncology 2019.

Maglennon GA, McIntosh P, Doorbar J. 2011. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology 414(2), 153-163.

Mak R, Van Renterghem L, Cuvelier C. 2004. Cervical smears and human papillomavirus typing in sex workers. Sexually transmitted infections 80(2), 118-120.

Maleki A, Ahmadnia E, Avazeh A, Mazloomzadeh S, Molaei B, Jalilvand A. 2015a. Prevalence of abnormal papanicolaou test results and related factors among women living in Zanjan, Iran. Asian Pac J Cancer Prev 16(16), 6935-6939.

Maleki A, Ahmadnia E, Avazeh A, Mazloomzadeh S, Molaei B, Jalilvand A. 2015b. Prevalence of abnormal papanicolaou test results and related factors among women living in Zanjan, Iran. Asian Pacific Journal of Cancer Prevention 16(16), 6935-6939.

Manisha S, Bagde N, Shrivastava D. 2017. Visual inspection of cervix with acetic acid: An alternative to cytology and colposcopy in early screening of cervical cancer in low-resource setup. Journal of Datta Meghe Institute of Medical Sciences University 12(1), 32.

Martin RE. 2000. Is it feasible for women to perform their own Pap smears? A research question in progress. Canadian Medical Association Journal 162(5), 666-667.

Maw RD, Reitano M, Roy M. 1998. An international survey of patients with genital warts: perceptions regarding treatment and impact on lifestyle. International journal of STD & AIDS 9(10), 571-578.

McBride AA. 2013. The papillomavirus E2 proteins. Virology 445(1-2), 57-79.

McBride AA, Warburton A. 2017. The role of integration in oncogenic progression of HPV-associated cancers. PLoS pathogens 13(4), e1006211.

McIntosh PB, Laskey P, Sullivan K, Davy C, Wang Q, Jackson DJ, Griffin HM, Doorbar J. 2010. E1^ E4-mediated keratin phosphorylation and ubiquitylation: A mechanism for keratin depletion in HPV16-infected epithelium. J Cell Sci 123(16), 2810-2822.

Mehta A, Ladola H, Kotadiya K, Edwin R, Patel V. 2013. Study of high risk cases for early detection of cervical cancer by Pap smear and VILI. NHL J Med Sci 2, 65-68.

Meiring TL, Salimo AT, Coetzee B, Maree HJ, Moodley J, Hitzeroth II, Freeborough M-J, Rybicki EP, Williamson A-L. 2012. Next-generation sequencing of cervical DNA detects human papillomavirus types not detected by commercial kits. Virology journal 9(1), 164.

Melnikow J, McGahan C, Sawaya GF, Ehlen T, Coldman A. 2009. Cervical intraepithelial neoplasia outcomes after treatment: long-term follow-up from the British Columbia Cohort Study. JNCI: Journal of the National Cancer Institute 101(10), 721-728.

Middleton K, Peh W, Southern S, Griffin H, Sotlar K, Nakahara T, El-Sherif A, Morris L, Seth R, Hibma M. 2003. Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. Journal of virology 77(19), 10186-10201.

Milligan SG, Veerapraditsin T, Ahamet B, Mole S, Graham SV. 2007. Analysis of novel human papillomavirus type 16 late mRNAs in differentiated W12 cervical epithelial cells. Virology 360(1), 172-181.

Modis Y, Trus BL, Harrison SC. 2002. Atomic model of the papillomavirus capsid. The EMBO journal 21(18), 4754-4762.

Morema EN, Atieli HE, Onyango RO, Omondi JH, Ouma C. 2014. Determinants of cervical screening services uptake among 18–49 year old women seeking services at the Jaramogi Oginga Odinga Teaching and Referral Hospital, Kisumu, Kenya. BMC Health Services Research 14(1), 335.

Mori S, Nakao S, Kukimoto I, Kusumoto-Matsuo R, Kondo K, Kanda T. 2011. Biased amplification of human papillomavirus DNA in specimens containing multiple human papillomavirus types by PCR with consensus primers. Cancer science 102(6), 1223-1227.

Morris BJ, Hankins CA. 2017. Effect of male circumcision on risk of sexually transmitted infections and cervical cancer in women. The Lancet Global Health 5(11), e1054-e1055.

Morris BJ, Gray RH, Castellsague X, Bosch FX, Halperin DT, Waskett JH, Hankins CA. 2011. The strong protective effect of circumcision against cancer of the penis. Advances in urology 2011.

Morrison B. 2014. Risk Factors and Prevalence of Penile Cancer: The University of the West Indies.

Moscicki A-B, Ma Y, Wibbelsman C, Darragh TM, Powers A, Farhat S, Shiboski S. 2010. Rate of and risks for regression of CIN-2 in adolescents and young women. Obstetrics and gynecology 116(6), 1373.

Mulhim, NKA-O, Mohammed, Saad Morsi AM. 2014. Knowledge about cervical cancer early warning signs and symptoms, risk factors and vaccination among students at a medical school in Al-Ahsa, Kingdom of Saudi Arabia. Asian Pacific Journal of Cancer Prevention 15(6), 2529-2532.

Münger K, Werness B, Dyson N, Phelps W, Harlow E, Howley P. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. The EMBO journal 8(13), 4099-4105.

Munoz N, Castellsagué X, de González AB, Gissmann L. 2006. HPV in the etiology of human cancer. Vaccine 24, S1-S10.

Munoz N, Bosch FX, Castellsagué X, Díaz M, De Sanjose S, Hammouda D, Shah KV, Meijer CJ. 2004. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. International journal of cancer 111(2), 278-285.

Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ. 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003(348), 518-527.

Muñoz N, Méndez F, Posso H, Molano M, Van Den Brule AJ, Ronderos M, Meijer C, Muñoz Á, Group INdCHS. 2004. Incidence, duration, and determinants of cervical human papillomavirus infection in a cohort of Colombian women with normal cytological results. The Journal of infectious diseases 190(12), 2077-2087.

Muñoz N, Franceschi S, Bosetti C, Moreno V, Herrero R, Smith JS, Shah KV, Meijer CJ, Bosch FX, Group IAfRoCMCCS. 2002. Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. The Lancet 359(9312), 1093-1101.

Nayar R, Wilbur DC. 2015. The pap test and Bethesda 2014. Acta cytologica 59(2), 121-132.

Naz MSG, Darooneh T, Fakari FR, kholosi Badr F, Hajizadeh F, Ozgoli G. 2018. The Relationship between Health Locus of Control and Iranian Women's Beliefs toward Pap Smear Screening. International Journal of Community Based Nursing and Midwifery 7(1), 43-51.

Nunes RAL, Morale MG, Silva GÁF, Villa LL, Termini L. 2018. Innate immunity and HPV: friends or foes. Clinics 73.

Oaknin A, de Corcuera ID, Rodríguez-Freixinós V, Rivera F, del Campo JM. 2012. SEOM guidelines for cervical cancer. Clinical and Translational Oncology 14(7), 516-519.

Oh HY, Kim MK, Seo S-S, Lee J-K. 2016. Association of combined tobacco smoking and oral contraceptive use with cervical intraepithelial neoplasia 2 or 3 in Korean women. Journal of Epidemiology 26(1), 22-29.

Organization WH. 2010. Report of the meeting on HPV vaccine coverage and impact monitoring, 16-17 November 2009, Geneva, Switzerland.

Origoni M, Cristoforoni P, Carminati G, Stefani C, Costa S, Sandri MT, Mariani L, Preti M. 2015. E6/E7 mRNA testing for human papilloma virus-induced high-grade cervical intraepithelial disease (CIN2/CIN3): a promising perspective. Ecancermedicalscience 9.

Oyervides-Munoz MA, Perez-Maya AA, Rodríguez-Gutierrez HF, Macias GSG, Fajardo-Ramirez OR, Trevino V, Barrera-Saldana HA, Garza-Rodriguez ML. 2018. Understanding the HPV integration and its progression to cervical cancer. Infection, Genetics and Evolution 61, 134-144.

Pai SI, Westra WH. 2009. Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. Annual Review of Pathological Mechanical Disease 4, 49-70.

Parkin DM, Bray F. 2006. The burden of HPV-related cancers. Vaccine 24, S11-S25.

Patel MM, Pandya AN, Modi J. 2011. Cervical Pap smear study and its utility in cancer screening, to specify the strategy for cervical cancer control. National Journal of Community Medicine 2(1), 49-51.

Pedroza-Saavedra A, Lam EW, Esquivel-Guadarrama F, Gutierrez-Xicotencatl L. 2010. The human papillomavirus type 16 E5 oncoprotein synergizes with EGF-receptor signaling to enhance cell cycle progression and the down-regulation of p27Kip1. Virology 400(1), 44-52.

Peplow PV, Chatterjee MP. 2013. A review of the influence of growth factors and cytokines in in vitro human keratinocyte migration. Cytokine 62(1), 1-21.

Pett MR, Herdman MT, Palmer RD, Yeo GS, Shivji MK, Stanley MA, Coleman N. 2006. Selection of cervical keratinocytes containing integrated HPV16 associates with episome loss and an endogenous antiviral response. Proceedings of the National Academy of Sciences 103(10), 3822-3827.

Pirog EC, Lloveras B, Molijn A, Tous S, Guimerà N, Alejo M, Clavero O, Klaustermeier J, Jenkins D, Quint WG. 2014. HPV prevalence and genotypes in different histological subtypes of cervical adenocarcinoma, a worldwide analysis of 760 cases. Modern Pathology 27(12), 1559.

Plenat F, Montagne K, Weinbreck N, Corby S, Champigneulle J, Antunes L, Bonnet C, Maire C, Monhoven N. 2006. Molecular consequences of fixation and tissue processing: the examples of nucleic acids and proteins. Pages 8-21. Annales de pathologie.

Plummer M, Peto J, Franceschi S, Cancer ICoESoC. 2012. Time since first sexual intercourse and the risk of cervical cancer. International journal of cancer 130(11), 2638-2644.

Qing C. 2017. The molecular biology in wound healing & non-healing wound. Chinese Journal of Traumatology 20(4), 189-193.

Quesnelle KM, Boehm AL, Grandis JR. 2007. STAT-mediated EGFR signaling in cancer. Journal of cellular biochemistry 102(2), 311-319.

Ranjeva SL, Baskerville EB, Dukic V, Villa LL, Lazcano-Ponce E, Giuliano AR, Dwyer G, Cobey S. 2017. Recurring infection with ecologically distinct HPV types can explain high prevalence and diversity. Proceedings of the National Academy of Sciences 114(51), 13573-13578.

Ratnam S, Coutlee F, Fontaine D, Bentley J, Escott N, Ghatage P, Gadag V, Holloway G, Bartellas E, Kum N. 2011. Aptima HPV E6/E7 mRNA test is as sensitive as Hybrid Capture

2 Assay but more specific at detecting cervical precancer and cancer. Journal of clinical microbiology 49(2), 557-564.

Raza S, Franceschi S, Pallardy S, Malik F, Avan B, Zafar A, Ali S, Pervez S, Serajuddaula S, Snijders P. 2010. Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. British journal of cancer 102(11), 1657.

Richards RM, Lowy DR, Schiller JT, Day PM. 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proceedings of the National Academy of Sciences 103(5), 1522-1527.

Richart RM. 1973. Cervical intraepithelial neoplasia. Pathol. Annu. 8, 301-328.

Rigoni-Stern D. 1842. Fatti statistici relativi alle malattie cancerose. Giorn Prog Patol Terap 2, 507-517.

Rijkaart D, Heideman D, Coupe V, Brink A, Verheijen R, Skomedal H, Karlsen F, Morland E, Snijders P, Meijer C. 2012. High-risk human papillomavirus (hrHPV) E6/E7 mRNA testing by PreTect HPV-Proofer for detection of cervical high-grade intraepithelial neoplasia and cancer among hrHPV DNA-positive women with normal cytology. Journal of clinical microbiology 50(7), 2390-2396.

Risau W. 1997. Mechanisms of angiogenesis. nature 386.

Roman A. 2006. The human papillomavirus E7 protein shines a spotlight on the pRB family member, p130. Cell Cycle 5(6), 567-568.

Rose PG, Bundy BN, Watkins EB, Thigpen JT, Deppe G, Maiman MA, Clarke-Pearson DL, Insalaco S. 1999. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. New England Journal of Medicine 340(15), 1144-1153.

Rosenberger S, Arce JD-C, Langbein L, Steenbergen RD, Rösl F. 2010. Alternative splicing of human papillomavirus type-16 E6/E6* early mRNA is coupled to EGF signaling via Erk1/2 activation. Proceedings of the National Academy of Sciences 107(15), 7006-7011.

Roura E, Castellsagué X, Pawlita M, Travier N, Waterboer T, Margall N, Bosch FX, De Sanjosé S, Dillner J, Gram IT. 2014. Smoking as a major risk factor for cervical cancer and pre-cancer: Results from the EPIC cohort. International journal of cancer 135(2), 453-466.

Rous P, Beard J. 1935. The progression to carcinoma of virus-induced rabbit papillomas (Shope). Journal of Experimental Medicine 62(4), 523-548.

Rozendaal L, Westerga J, Van der Linden J, Walboomers J, Voorhorst F, Risse E, Boon M, Meijer C. 2000. PCR based high risk HPV testing is superior to neural network based screening for predicting incident CIN III in women with normal cytology and borderline changes. Journal of clinical pathology 53(8), 606-611.

Ryndock EJ, Meyers C. 2014. A risk for non-sexual transmission of human papillomavirus?: Taylor & Francis.

Sabeena S, Bhat P, Kamath V, Arunkumar G. 2017. Possible non-sexual modes of transmission of human papilloma virus. Journal of Obstetrics and Gynaecology Research 43(3), 429-435.

Sachan PL, Singh M, Patel ML, Sachan R. 2018. A study on cervical cancer screening using pap smear test and clinical correlation. Asia-Pacific journal of oncology nursing 5(3), 337.

Salati SA, Al Kadi A. 2012. Anal cancer–a review. International journal of health sciences 6(2), 206.

Salih MM, Alhag FTES, Khalifa MA, El Nabi AH. 2017. Cervical cytopathological changes among women with vaginal discharge attending teaching hospital. Journal of Cytology 34(2), 90.

Salman KF. 2012. Health beliefs and practices related to cancer screening among Arab Muslim women in an urban community. Health Care for Women International 33(1), 45-74.

Samanta A, Ghosh S, Mukherjee S. 2011. Prevalence and health-seeking behavior of reproductive tract infection/sexually transmitted infections symptomatics: A cross-sectional study of a rural community in the Hooghly district of West Bengal. Indian Journal of Public Health 55(1), 38.

Sanger F. 1992. DNA sequencing with chain-terminating inhibitors. 1977. Biotechnology 24, 104-108.

Santesso N, Mustafa RA, Wiercioch W, Kehar R, Gandhi S, Chen Y, Cheung A, Hopkins J, Khatib R, Ma B. 2016. Systematic reviews and meta-analyses of benefits and harms of cryotherapy, LEEP, and cold knife conization to treat cervical intraepithelial neoplasia. International Journal of Gynecology & Obstetrics 132(3), 266-271.

Saraiya M, Steben M, Watson M, Markowitz L. 2013. Evolution of cervical cancer screening and prevention in United States and Canada: implications for public health practitioners and clinicians. Preventive medicine 57(5), 426-433.

Saraiya M, Ahmed F, Krishnan S, Richards TB, Unger ER, Lawson HW. 2007. Cervical cancer incidence in a prevaccine era in the United States, 1998–2002. Obstetrics & Gynecology 109(2), 360-370.

Sarkola ME, Grénman SE, Rintala MA, Syrjänen KJ, Syrjänen SM. 2008. Human papillomavirus in the placenta and umbilical cord blood. Acta Obstetricia et Gynecologica Scandinavica 87(11), 1181-1188.

Satterwhite CL, Torrone E, Meites E, Dunne EF, Mahajan R, Ocfemia MCB, Su J, Xu F, Weinstock H. 2013. Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. Sexually transmitted diseases 40(3), 187-193.

Schellekens MC, Dijkman A, Aziz MF, Siregar B, Cornain S, Kolkman-Uljee S, Peters LA, Fleuren GJ. 2004. Prevalence of single and multiple HPV types in cervical carcinomas in Jakarta, Indonesia. Gynecologic oncology 93(1), 49-53.

Schenk T, Brandstetter T, Zur Hausen A, Alt-Mörbe J, Huzly D, Rühe J. 2009. Performance of a polymer-based DNA chip platform in detection and genotyping of human papillomavirus in clinical samples. J Clin Microbiol 47(5), 1428-1435.

Schiffman M, Solomon D. 2003. Findings to date from the ASCUS-LSIL Triage Study (ALTS). Archives of pathology & laboratory medicine 127(8), 946-949.

Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. 2007. Human papillomavirus and cervical cancer. The Lancet 370(9590), 890-907.

Schiller JT, Lowy DR. 2012. Understanding and learning from the success of prophylactic human papillomavirus vaccines. Nature Reviews Microbiology 10(10), 681.

Schiller JT, Day PM, Kines RC. 2010. Current understanding of the mechanism of HPV infection. Gynecologic oncology 118(1), S12-S17.

Schockaert S, Poppe W, Arbyn M, Verguts T, Verguts J. 2008. Incidence of vaginal intraepithelial neoplasia after hysterectomy for cervical intraepithelial neoplasia: a retrospective study. American journal of obstetrics and gynecology 199(2), 113. e111-113. e115.

Schultz G, Clark W, Rotatori DS. 1991. EGF and TGF-α in wound healing and repair. Journal of cellular biochemistry 45(4), 346-352.

Schwartz S. 2008. HPV-16 RNA processing. Front Biosci 13, 5880-5891.

Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A, zur Hausen H. 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. nature 314(6006), 111.

Scott ME, Ma Y, Farhat S, Moscicki AB. 2015. Expression of nucleic acid-sensing T oll-like receptors predicts HPV 16 clearance associated with an E 6-directed cell-mediated response. International journal of cancer 136(10), 2402-2408.

Seeger MA, Paller AS. 2015. The roles of growth factors in keratinocyte migration. Advances in wound care 4(4), 213-224.

Shafiq M, Ali SH. 2006. Sexually transmitted infections in Pakistan. Lancet Infectious Diseases 6(6), 321-322.

Shahsiah R, Khademalhosseini M, Mehrdad N, Ramezani F, Nadji SA. 2011. Human papillomavirus genotypes in Iranian patients with cervical cancer. Pathology-Research and Practice 207(12), 754-757.

Shanmugham D, Vijay A, Rangaswamy T. 2014. Colposcopic Evaluation of Patients with Persistent Inflammatory Pap Smear. Significance atypical squamous cells of undetermined significance (ASCUS) 3, 1-5.

Shaw PA. 2000. The history of cervical screening I: the pap. Test. Journal SOGC 22(2), 110-114.

Shaw R, Beasley N. 2016. Aetiology and risk factors for head and neck cancer: United Kingdom National Multidisciplinary Guidelines. The Journal of Laryngology & Otology 130(S2), S9-S12.

Sheardown H, Cheng YL. 1996. Tear EGF concentration following corneal epithelial wound creation. J Ocul Pharmacol Ther 12(3), 239-243.

Shedlock AM, Haygood MG, Pietsch TW, Bentzen P. 1997. Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. Biotechniques 22(3), 394-400.

Shen-Gunther J, Yu X. 2011. HPV molecular assays: defining analytical and clinical performance characteristics for cervical cytology specimens. Gynecologic oncology 123(2), 263-271.

Shendure J, Ji H. 2008. Next-generation DNA sequencing. Nature biotechnology 26(10), 1135.

Shi S-R, Datar R, Liu C, Wu L, Zhang Z, Cote RJ, Taylor CR. 2004. DNA extraction from archival formalin-fixed, paraffin-embedded tissues: heat-induced retrieval in alkaline solution. Histochemistry and cell biology 122(3), 211-218.

Shirakata Y. 2010. Regulation of epidermal keratinocytes by growth factors. Journal of dermatological science 59(2), 73-80.

Shope RE, Hurst EW. 1933. Infectious papillomatosis of rabbits: with a note on the histopathology. Journal of Experimental Medicine 58(5), 607-624.

Shukla S, Bharti AC, Mahata S, Hussain S, Hedau S, Sharma R, Pillai MR, Krishna S, Chiplunkar S, Tongaonkar H. 2010. Application of a multiplex PCR to cervical cells collected by a paper smear for the simultaneous detection of all mucosal human papillomaviruses (HPVs) and typing of high-risk HPV types 16 and 18. Journal of medical microbiology 59(11), 1303-1310.

Siddiqa A, Zainab M, Qadri I, Bhatti MF, Parish JL. 2014. Prevalence and genotyping of high risk human papillomavirus in cervical cancer samples from Punjab, Pakistan. Viruses 6(7), 2762-2777.

Sideri M, Jones RW, Wilkinson EJ, Preti M, Heller DS, Scurry J, Haefner H, Neill S. 2005. Squamous vulvar intraepithelial neoplasia: 2004 modified terminology, ISSVD Vulvar Oncology Subcommittee. The Journal of reproductive medicine 50(11), 807-810.

Siegel R, Ma J, Zou Z, Jemal A. 2014. Cancer statistics, 2014. CA: a cancer journal for clinicians 64(1), 9-29.

Singla AA, Komesaroff P. 2018. Self-collected Pap smears may provide an acceptable and effective method of cervical cancer screening. Health Science Reports 1(5), 33.

Sinno H, Prakash S. 2013. Complements and the wound healing cascade: an updated review. Plastic surgery international 2013.

Smith CJ, Osborn AM. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS microbiology ecology 67(1), 6-20.

Smith EM, Parker MA, Rubenstein LM, Haugen TH, Hamsikova E, Turek LP. 2010. Evidence for vertical transmission of HPV from mothers to infants. Infectious diseases in obstetrics and gynecology 2010.

Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell CR, Heiner C, Kent SB, Hood LE. 1986. Fluorescence detection in automated DNA sequence analysis. Nature 321(6071), 674.

Solomon D. 1989. The 1988 Bethesda system for reporting cervical/vaginal cytological diagnosis. JAMA 262, 931-934.

Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright Jr T. 2002. The 2001 Bethesda System: terminology for reporting results of cervical cytology. Jama 287(16), 2114-2119.

Song D, Li H, Li H, Dai J. 2015. Effect of human papillomavirus infection on the immune system and its role in the course of cervical cancer. Oncology letters 10(2), 600-606.

Spink KM, Laimins LA. 2005. Induction of the human papillomavirus type 31 late promoter requires differentiation but not DNA amplification. Journal of virology 79(8), 4918-4926.

Srinivasan M, Sedmak D, Jewell S. 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 161(6), 1961-1971.

Stanley M. 2009. Immune responses to human papilloma viruses. Indian Journal of Medical Research 130(3), 266.

Stanley M. 2010. HPV-immune response to infection and vaccination. Infectious agents and cancer 5(1), 19.

Stanley M, Pinto LA, Trimble C. 2012. Human papillomavirus vaccines–immune responses. Vaccine 30, F83-F87.

Stanley MA. 2012. Epithelial cell responses to infection with human papillomavirus. Clinical microbiology reviews 25(2), 215-222.

Stanley MA, Browne HM, Appleby M, Minson AC. 1989. Properties of a non-tumorigenic human cervical keratinocyte cell line. International journal of cancer 43(4), 672-676.

Stasik S, Schuster C, Ortlepp C, Platzbecker U, Bornhäuser M, Schetelig J, Ehninger G, Folprecht G, Thiede C. 2018. An optimized targeted next-generation sequencing approach for sensitive detection of single nucleotide variants. Biomolecular detection and quantification 15, 6-12.

Stier EA, Sebring MC, Mendez AE, Ba FS, Trimble DD, Chiao EY. 2015. Prevalence of anal human papillomavirus infection and anal HPV-related disorders in women: a systematic review. American journal of obstetrics and gynecology 213(3), 278-309.

Stratton KL, Culkin DJ. 2016. A contemporary review of HPV and penile cancer. Oncology 30(3).

Strohl AE, Mendoza G, Ghant MS, Cameron KA, Simon MA, Schink JC, Marsh EE. 2015. Barriers to prevention: knowledge of HPV, cervical cancer, and HPV vaccinations among African American women. American Journal of Obstetrics and Gynecology 212(1), 65.

Stumbar SE, Stevens M, Feld Z. 2019. Cervical cancer and its precursors: a preventative approach to screening, diagnosis, and management. Primary Care: Clinics in Office Practice 46(1), 117-134.

Tang S, Tao M, McCoy JP, Zheng Z-M. 2006. The E7 oncoprotein is translated from spliced E6* I transcripts in high-risk human papillomavirus type 16-or type 18-positive cervical cancer cell lines via translation reinitiation. Journal of virology 80(9), 4249-4263.

Tardif KD, Simmon KE, Kommedal Ø, Pyne MT, Schlaberg R. 2013. Sequencing-based genotyping of mixed human papillomavirus infections by use of RipSeq software. J Clin Microbiol 51(4), 1278-1280.

Tawe L, Grover S, Narasimhamurthy M, Moyo S, Gaseitsiwe S, Kasvosve I, Paganotti GM. 2018. Molecular detection of human papillomavirus (HPV) in highly fragmented DNA from cervical cancer biopsies using double-nested PCR. MethodsX.

Tayyeb R, Khawaja N, Malik N. 2003. Comparison of visual inspection of cervix and Pap smear for cervical cancer screening. Journal of the College of Physicians and Surgeons 13(4), 201-203.

Tenti P, Zappatore R, Migliora P, Spinillo A, Belloni C, Carnevali L. 1999. Perinatal transmission of human papillomavirus from gravidas with latent infections. Obstetrics & Gynecology 93(4), 475-479.

Terai M, Burk RD. 2002. Felis domesticus papillomavirus, isolated from a skin lesion, is related to canine oral papillomavirus and contains a 1· 3 kb non-coding region between the E2 and L2 open reading frames. Journal of general virology 83(9), 2303-2307.

Thierry F. 2009. Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in cervical carcinoma. Virology 384(2), 375-379.

Thomas M, Myers MP, Massimi P, Guarnaccia C, Banks L. 2016. Analysis of multiple HPV E6 PDZ interactions defines type-specific PDZ fingerprints that predict oncogenic potential. PLoS pathogens 12(8), e1005766.

Tipu HN, Shabbir A. 2015. Evolution of DNA sequencing. J Coll Physicians Surg Pak 25(3), 210-215.

Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, Yahata Y, Dai X, Tohyama M, Nagai H. 2005. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. The journal of immunology 175(7), 4662-4668.

Topcul M, Cetin I. 2014. Endpoint of cancer treatment: Targeted therapies. Asian Pac J Cancer Prev 15(11), 4395-4403.

Valencia C, Bonilla-Delgado J, Oktaba K, Ocádiz-Delgado R, Gariglio P, Covarrubias L. 2008. Human papillomavirus E6/E7 oncogenes promote mouse ear regeneration by increasing the rate of wound re-epithelization and epidermal growth. Journal of Investigative Dermatology 128(12), 2894-2903.

Van Doorslaer K, McBride AA. 2016. Molecular archeological evidence in support of the repeated loss of a papillomavirus gene. Scientific reports 6, 33028.

Van Doorslaer K, Li Z, Xirasagar S, Maes P, Kaminsky D, Liou D, Sun Q, Kaur R, Huyen Y, McBride AA. 2016. The Papillomavirus Episteme: a major update to the papillomavirus sequence database. Nucleic acids research 45(D1), D499-D506.

Verdoodt F, Szarewski A, Halfon P, Cuschieri K, Arbyn M. 2013. Triage of women with minor abnormal cervical cytology: Meta-analysis of the accuracy of an assay targeting messenger ribonucleic acid of 5 high-risk human papillomavirus types. Cancer cytopathology 121(12), 675-687.

Villa LL, Denny L. 2006a. CHAPTER 7 Methods for detection of HPV infection and its clinical utility. International Journal of Gynecology & Obstetrics 94, S71-S80.

Villa LL, Denny L. 2006b. Methods for detection of HPV infection and its clinical utility. International Journal of Gynecology & Obstetrics 94, S71-S80.

Vu M, Yu J, Awolude OA, Chuang L. 2018. Cervical cancer worldwide. Current Problems in Cancer 42(5), 457-465.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. The Journal of pathology 189(1), 12-19.

Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P. 2004. Functional analysis of the human papillomavirus type 16 E1∧E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. Journal of virology 78(2), 821-833.

Wardak S. 2016. Human Papillomavirus (HPV) and cervical cancer. Medycyna doswiadczalna i mikrobiologia 68(1), 73-84.

Wechsler EI, Wang Q, Roberts I, Pagliarulo E, Jackson D, Untersperger C, Coleman N, Griffin H, Doorbar J. 2012. Reconstruction of human papillomavirus type 16-mediated early-stage neoplasia implicates E6/E7 deregulation and the loss of contact inhibition in neoplastic progression. Journal of virology 86(11), 6358-6364.

Wentzensen N, Arbyn M, Berkhof J, Bower M, Canfell K, Einstein M, Farley C, Monsonego J, Franceschi S. 2017. Eurogin 2016 Roadmap: How HPV knowledge is changing screening practice. International Journal of Cancer 140(10), 2192-2200.

Werner S, Grose R. 2003a. Regulation of wound healing by growth factors and cytokines. Physiological reviews 83(3), 835-870.

Werner S, Grose R. 2003b. Regulation of wound healing by growth factors and cytokines. Physiol Rev 83(3), 835-870.

Werner S, Krieg T, Smola H. 2007. Keratinocyte-fibroblast interactions in wound healing. J Invest Dermatol 127(5), 998-1008.

White EA, Howley PM. 2013. Proteomic approaches to the study of papillomavirus–host interactions. Virology 435(1), 57-69.

Widdice L, Ma Y, Jonte J, Farhat S, Breland D, Shiboski S, Moscicki A-B. 2013. Concordance and transmission of human papillomavirus within heterosexual couples observed over short intervals. The Journal of infectious diseases 207(8), 1286-1294.

Wilson SE, Liang Q, Kim WJ. 1999. Lacrimal gland HGF, KGF, and EGF mRNA levels increase after corneal epithelial wounding. Invest Ophthalmol Vis Sci 40(10), 2185-2190.

Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee S-K, Kuypers JM, Koutsky LA. 2005. Development and duration of human papillomavirus lesions, after initial infection. The Journal of infectious diseases 191(5), 731-738.

Winer RL, Hughes JP, Feng Q, Xi LF, Cherne S, O'Reilly S, Kiviat NB, Koutsky LA. 2010. Detection of genital HPV types in fingertip samples from newly sexually active female university students. Cancer Epidemiology and Prevention Biomarkers 19(7), 1682-1685.

Wolf M, Garcea RL, Grigorieff N, Harrison SC. 2010. Subunit interactions in bovine papillomavirus. Proceedings of the National Academy of Sciences 107(14), 6298-6303.

Woodby B, Scott M, Bodily J. 2016. The interaction between human papillomaviruses and the stromal microenvironment. Pages 169-238. Progress in molecular biology and translational science, vol. 144 Elsevier.

Woodworth CD, Diefendorf LP, Jette DF, Mohammed A, Moses MA, Searleman SA, Stevens DA, Wilton KM, Mondal S. 2011. Inhibition of the epidermal growth factor

receptor by erlotinib prevents immortalization of human cervical cells by Human Papillomavirus type 16. Virology 421(1), 19-27.

Workowski KA, Bolan GA. 2015. Sexually transmitted diseases treatment guidelines, 2015. MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports 64(RR-03), 1.

Xi LF, Koutsky LA, Castle PE, Edelstein ZR, Meyers C, Ho J, Schiffman M. 2009. Relationship between cigarette smoking and human papilloma virus types 16 and 18 DNA load. Cancer Epidemiology and Prevention Biomarkers 18(12), 3490-3496.

Yasmin S, Mukherjee A. 2012. A cyto-epidemiological study on married women in reproductive age group (15-49 years) regarding reproductive tract infection in a rural community of West Bengal. Indian Journal of Public Health 56(3), 204.

Yeung CA, Tsang T, Yau P, Kwok T. 2011. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. Oncogene 30(21), 2401.

Yousuf S, Syed S, Moazzam A, Lucky MH. 2010. Frequency of high risk human papillomavirus types in squamous cell carcinoma of cervix among women. J Pak Med Assoc 60(3), 193.

Zaravinos A, Mammas IN, Sourvinos G, Spandidos DA. 2009. Molecular detection methods of human papillomavirus (HPV). The International journal of biological markers 24(4), 215-222.

Zekan J, Skerlev M, Milić L, Karelović D. 2014. Human papillomavirus-related diseases of the female lower genital tract: oncogenic aspects and molecular interaction. Collegium antropologicum 38(2), 779-786.

Zhai L, Tumban E. 2016. Gardasil-9: A global survey of projected efficacy. Antiviral research 130, 101-109.

Zhang D, Li T, Chen L, Zhang X, Zhao G, Liu Z. 2017. Epidemiological investigation of the relationship between common lower genital tract infections and high-risk human papillomavirus infections among women in Beijing, China. PloS one 12(5), e0178033.

Zhao F-H, Lewkowitz AK, Chen F, Lin MJ, Hu S-Y, Zhang X, Pan Q-J, Ma J-F, Niyazi M, Li C-Q. 2012. Pooled analysis of a self-sampling HPV DNA test as a cervical cancer primary screening method. Journal of the National Cancer Institute 104(3), 178-188.

Zheng Z-M, Baker CC. 2006a. Papillomavirus genome structure, expression, and post-transcriptional regulation. Frontiers in bioscience: a journal and virtual library 11, 2286.

Zheng ZM, Baker CC. 2006b. Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci 11, 2286-2302.

Zhong P, Li J, Gu Y, Liu Y, Wang A, Sun Y, Lu L. 2015. P16 and Ki-67 expression improves the diagnostic accuracy of cervical lesions but not predict persistent high risk human papillomavirus infection with CIN1. International journal of clinical and experimental pathology 8(3), 2979.

- Zhou XH, Shi YF, Wang LJ, Liu M, Li F. 2017. Distribution characteristics of human papillomavirus infection: a study based on data from physical examination. Asian Pacific journal of cancer prevention: APJCP 18(7), 1875.
- Zur Hausen H. 1976. Condylomata acuminata and human genital cancer. Cancer Res 36(2 pt 2), 794.
- zur Hausen H. 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2(5), 342-350.