Genotype Distribution and Sero-epidemiology of Human Papillomavirus (HPV) in Female Population of Bangladesh

A THESIS SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF DHAKA
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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled **"Genotype Distribution and Sero-epidemiology of Human Papillomavirus (HPV) in Female Population of Bangladesh"** submitted by **Shahana Sharmin**, Registration No. 206/2014-2015, carried out her research under our supervision. This is further to certify that it is an original work and suitable for partial fulfilment of the degree of Doctor of Philosophy (PhD) in Microbiology, University of Dhaka.

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(Supervisor)

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DECLARATION

I do hereby declare that the work submitted as a thesis entitled: **"Genotype Distribution and Sero-epidemiology of Human Papillomavirus (HPV) in Female Population of Bangladesh"** to the Department of Microbiology, University of Dhaka for the degree of Doctor of Philosophy (PhD) by the results of my own investigations and carried out under supervision of Prof. Dr. Mahmuda Yasmin, Professor, Department of Microbiology, University of Dhaka. The research work has not previously been submitted for any degree.

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Shahana Sharmin

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Registration No.: 206

Session: 2014-2015

Dedicated to

My beloved parents, my husband and my sons

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ABSTRACT

Cervical cancer is one of the most prevalent cancer that develop in the cervix of the women worldwide. Annually 266,000 deaths are reported from 528,000 new cases of cervical cancer, which approximately account for 8% mortality of all female cancer patients. The most severe risk factor for cervical cancer till now is considered as an infection with specific human papillomavirus (HPV) types. The socioeconomic conditions of Bangladesh are constantly being threatened by an increasing concern regarding to the high mortality rate from this disease. Although several researches related to the epidemiology, HPV vaccination, and to some extent treatment protocols have been carried out in our country, any research regarding the prevalence and genotyping of HPV, on serology to detect potential biomarkers and on mutation profiling of several specific genes in cervical cancer in Bangladesh is yet to be done.

As we know that different types of HPVs are involved in infection, so the genotyping might reveal the high risk factors in both cervical cancer patient and apparently healthy women. It has been found that in case of cervical cancer 113 patients out of 115 (98.26%) are infected with HPV whereas 121 out of 410 (29.5%) apparently healthy women are infected with HPV. However, in this study we have presented that the age of women (Significant, p value <0.05), early exposure to sexual intercourse (Significant as p value <0.05), early pregnancy, multiple sexual partner, socioeconomic status (significant as p value <0.05), use of contraceptives, husband's occupation (Significant as p value <0.5) and the education level of the patient (Significant as p value < 0.05) etc. are among the several risk factors. Also we attempted to find out the responsible genotypes of HPV for the infection which later on might cause cervical cancer. Among the different types, this might be said that most prevalent genotype is HPV type 16 (53.7%) and HPV type 18 (19.06%) and their combinations (26.27%). Furthermore it has been observed that, apart from the HPV type 16 and type 18, also some other HPV types like type 6, type 62, type 69, type 31, type 33, type 90, and type 70 might also be responsible.

In case of sera samples this study justifies some of the goals to find out the potential biomarkers considering HMGB1 (High mobility group box 1), CEA (Carcinoembryonic antigen). Serpin B3/SCCA Squamous cell carcinoma antigen) and

Cytokeratin fragment 21-1 proteins, where it has been found that CEA was the prevalent protein to be shown in most of the patient's sera. Here, it also has been tried to detect possible correlations of antibody against HPV type 6, type 11, type 16 and type 18 in case of disease progression.

Finally, among the high number of genes involved in different signal transduction and cell growth regulation pathways, five different genomic regions within the top three most frequently mutated genes in COSMIC database with a key role in the development of cervical cancers were selected to study mutation frequency in our patients. These genes are *EGFR* (Epidermal Growth Factor Receptor), *KRAS* (Kirsten rat sarcoma), and *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha). In total, 39 mutations were found in 28 patient samples. Eleven different mutations (23.91%) were found in amplified *EGFR* gene fragments, among which 1 was common in seven patient samples. On the other hand, twenty-four different mutations (52.17%) were found in *PIK3CA* gene fragment amplicons, among which 2 were found in more than 1 patient. Four mutations (8.7%) were found in *KRAS* gene fragment amplified products. It has also been found that except for *KRAS*, the frequency of observed mutations in our patients is higher than those reported earlier in other parts of the world.

Our study finally summarizes that in case of Bangladesh the most considerable prevalent type of HPV might be HPV type 16 and type 18 and also to some extent their combinations. If serological study could be done further comparing with the control patient than established diagnostic tool could be generated. And also after the mutation profile study. The study can be used as a basis to build a mutation database for cervical cancer in Bangladesh. With the possibility of further exploration can be oriented towards establishing future diagnostics, personalized medicine decisions, and other pharmaceutical applications for specific cancer subtypes.

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
AJCC	American Joint Committee on Cancer
AML	Acute Myeloid Leukemia
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CBC	Complete Blood Count
CIN	Cervical Intraepithelial Neoplasia
CML	Chronic Myeloid Leukemia
CT	Computed Tomography
DES	Diethylstilbestrol
EBV	Epstein Barr Virus
ECC	Endocervical Curettage
ECG	Electro Cardiogram
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FIGO	International Federation of Gynecology and Obstetrics
GAVI	Global Alliance for Vaccines and Immunization
GDP	Guanosine diphosphate
GMT	Geometric Mean Antibody Titers
GTP	Guanosine triphosphate
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HHV8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus

HPV	Human Papilloma Virus
HTLV-1	Human T-Lymphotropic Virus Type 1
IMRT	Intensity-Modulated Radiation Therapy
KRAS	Kirsten Rat Sarcoma
LEEP	Loop Electrosurgical Excision Procedure
MCPyV	Merkel Cell Polyomavirus
MRI	Magnetic Resonance Imaging
MTBE	Methyl tert-butyl ether
NCBI	National Center for Biotechnology Information
NCCN	National Comprehensive Cancer Network
NHS	National Health Service
NICHR	National Institute of Cancer Research and Hospital
NIH	National Institute of Health
OCs	Oral Contraceptives
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFOA	Perfluorooctanoic acid
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PIK3CA	Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha
PtdIns	Phosphatidylinositols
SC	Squamocolumnar
TNM	Tumor Node Metastasis
TSG	Tumor Suppressor Genes
US FDA	United States Food and Drug Administration
VEGF	Vascular Endothelial Growth Factor

VIA Visual Inspection With Acetic Acid

VLPs Virus Like Particles

WHO World Health Organization

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Cervical cancer is a type of gynecologic cancer that develops in the cervix, which is a part of the uterus. Worldwide, cervical cancer was the fourth most common cancer among females in 2012 [1]. There was a report estimating 528,000 new cases of cervical cancer, of which around 85% occurred in less developed regions [1]. Around 266,000 deaths occur worldwide due to cervical cancer, accounting for 8% of all female cancer deaths [1]. In 2014, an estimated 12,578 women in the United States were diagnosed with cervical cancer; among them, 4115 women died of the disease [2]. The widespread use of cervical cancer screening programs has dramatically reduced rates of cervical cancer in developed countries [3].

Infection with Human papillomavirus (HPV) is the most important risk factor for cervical cancer [4]. In humans, specific papillomavirus types have been associated with over 99% of cervical cancer biopsies [5]. It is a common virus that can be sexually transmitted. Sexually active women can harbor HPV infection which is highly contagious to their partner [6]. HPV belongs to papillomavirus family and is a small, icosahedral, non-enveloped DNA virus [7]. HPV show tropism for stratified squamous epithelium. More than 200 genotypes of HPV have been found and were classified into two groups named the oncogenic and the non-oncogenic group [8]. The oncogenic group is again subdivided into two classes: high risk and low-risk group. Only 15 subtypes are identified in high-risk groups. They can cause neoplastic changes to the cervical epithelium. Globally, 75% of cervical cancer cases are caused by HPV types 16 and 18, while 31 and 45 are the causes of another 10% [9].

Besides HPV, multiple factors such as cigarette smoking (both active and passive), long-term use of oral contraceptives, multiple pregnancies, low socioeconomic status, being immunocompromised, multiple sexual partners are associated with increased risk of cervical cancer [10].

The risk of cancerous or precancerous lesion of the cervix and perineum is reduced with the use of two HPV vaccines named Gardasil and Cervarix which are effective between 92% and 100% against HPV 16 and 18 up to at least 8 years. The vaccines minimize the risk by about 93% and 62% respectively [11].

Cervical cancer, like other cancers, is a disorder of cell growth regulation. Different type of changes in genetic materials that can occur in a cell includes aneuploidy,

polymorphism, mosaicism, mutation etc. Mutation may be missense, nonsense, insertion, deletion, duplication, frameshift mutation and repeat expansion [12, 13]. These mutations are considered as significant when they occur in genes which control different signal transduction pathways. This is because these pathways associated with cell growth, cell cycle control, cell differentiation, apoptosis etc. There are specific genes which regulate cell growth and differentiation. Altered function of these genes leads a normal cell to transform into a cancer one [14].

Among the high number of genes involved in different signal transduction and cell growth regulation pathways, some are of special interest. Mutations in these genes play a key role in the development of different cancers. These genes are *EGFR* (Epidermal Growth Factor Receptor), *KRAS* (Kirsten rat sarcoma), and *PIK3CA* (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha). The protein product of *EGFR* gene is a receptor for members of the epidermal growth factor family (EGF family) of extracellular protein ligands [15]. *KRAS* gene which is a proto-oncogene corresponding to the oncogene that was first identified in Kirsten rat sarcoma virus [16] and its protein product is a GTPase that is an early player in many signal transduction pathways. Protein product of *PIK3CA* (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha) gene uses ATP to phosphorylate phosphatidylinositols (PtdIns), PtdIns4P and PtdIns P2.

The aim of this project is to find out mutation of any of these genes in cancerous tissue of cervical carcinoma patients in Bangladesh and to rule out the significance of these mutations in developing the disease as well.

1.2 Literature review

1.2.1 Human Papillomavirus (HPV)

Human Papillomavirus (HPV) is a DNA virus that belongs to papillomaviridae family. The virus is capable of infecting humans. They infect keratinocytes of the skin or mucous membranes. Most HPV infections are subclinical and do not show any symptoms. But sometimes in some people subclinical infections become clinical and may cause benign tumors or cancers of cervix, vulva, vagina, penis and anus [17]. HPV is one of the most common causes of sexually transmitted infection in both men

and women worldwide [18]. In particular, HPV-16 and HPV-18 are known to cause ~70% of cervical cancer [19].

1.2.2 Discovery of HPV

HPV was first identified during 1950s by a group of scientists from different countries. (Other characteristics, common associations and common strains of the virus were discovered later during 1970s and 1980s). Stefania Ginsburg-Jablonska, a Polish physician and dermatologist first discovered the connection between HPV and skin cancer in 1972. In 1977, Harald zur Hausen hypothesized that HPV plays an important role in the cause of cervical cancer [20]. In 1983 and 1984 zur Hausen and his collaborators identified HPV16 and HPV18 in cervical cancer [21].

1.2.3 Virion structure of HPV

The virions of HPV consist of a non-enveloped capsid of icosahedral symmetry. The capsid has a diameter of 40-55 nm. The capsid consists of 72 capsomers in skew arrangement. The surface appears rough and the surface projections are small [22]. The capsid consists of two structural proteins, the major capsid protein (L1) is about 55 kd in size and represents about 80% of the total viral protein and a minor protein (L2) has a molecular size of about 70 kd [23].

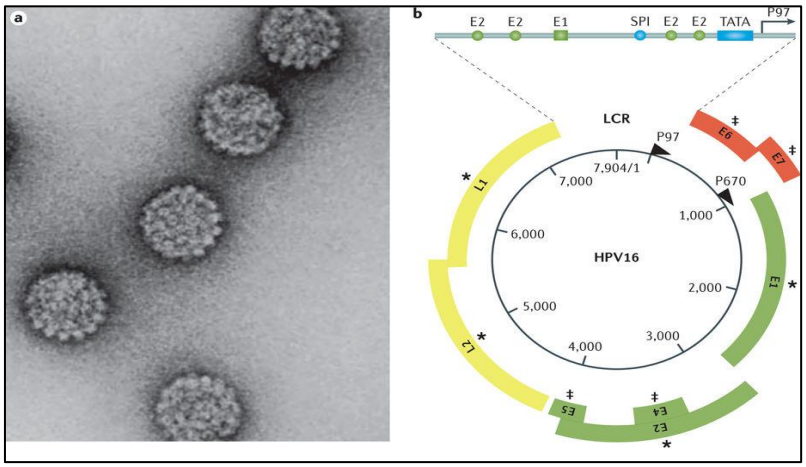

Figure 1.1: Human Papilloma Virus. a) Human papillomavirus (HPV) particles (55 nm in diameter) are shown in the negatively stained transmission electron micrograph. b) The genome organization typical of the high-risk Alpha HPV types illustrated as HPV16. Source: [24]

In addition, analysis of proteins in the virus particle has shown that the viral DNA is associated with cellular histones to form a chromatin-like complex [25].

1.2.4 Genome organization of HPV

The genetic material of HPV exists in the form of a double-stranded, circular DNA which is consists of 7200–8000 base pairs [26]. The genome is organized into three segments; early region (E) which comprises E1, E2, E4–E7 and represents 50% of the genome, the late region (L) consisting of L1 and L2 which represents 40% of the genome and a genomic regulatory region known as LCR (10% of the genome) [27].

All encoding protein fragments are located on a single DNA strand. These DNA fragments described as ORF (Open Reading Form) can be divided into early and late depending on the time of viral DNA replication occurrence [28].

Early fragments are involved in the regulation of DNA replication (E1, E2) transcription (E2) and cell transformation (E5, E6, E7) and late fragments encode structural proteins of the virion [29]. The L1 ORF is the most conserved region within the genome. So it is used for the identification of new papilloma-virus types. A new papillomavirus isolate is recognized if the complete genome has been cloned and the DNA sequence of the L1 ORF differs by more than 10% from the closest known type. Differences in homology ranging between 2% and 10% define a subtype and those of less than 1% define a variant [28].

The upstream region contains the regulatory sequences for transcription, the shared N-terminal sequence for the early proteins, and the origin of replication. The 400 to 1,000 bp noncoding part of upstream regulatory region which has been referred to as the noncoding region, the long control region (LCR), or the upper regulatory region. This region contains the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the ORFs. This region also contains the highest degree of variation in the viral genome [18].

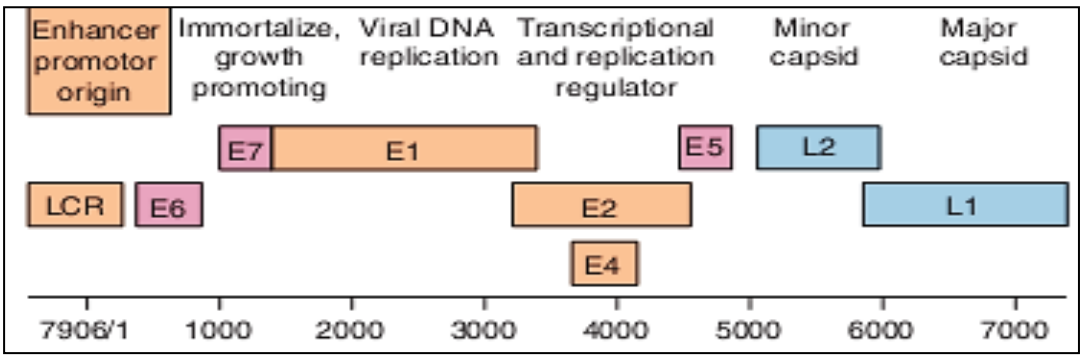

Figure 1.2: Genome of human papillomavirus type 16. Source: [30]

1.2.5 Proteins encoded by HPV genome and their role in carcinoma

E1 protein binds DNA at ori and promotes viral DNA replication helicase activity (like T antigen of SV 40). It is also a repressive agent in transcription and inhibits DNA replication, maintaining the episomal copies number within the cell at the same level [31, 32].

E2 protein combines with E1 protein and jointly they initiate. It is also responsible for coding proteins which regulate viral DNA transcription [32]. E2 also plays an important role in cell transformation, initiating and inhibiting apoptosis, transcriptional regulation, and in the modulation of the immortalizing and transformation potential of HPV [31]. E2 inactivation affects the development of tumor lesions by promoting the expression of E6 and E7, and active E2 inhibits the transcription of E6 and E7, causing an increase in p53 expression and apoptosis of the infected cells. The two proteins are essential in maintaining the replication of the virus and synthesis of the genes through the course of the differentiation process of the epithelium. They are also necessary for the virus to complete its replicable cycle [33].

E4 protein is a cytoplasmic protein disturbing the structural framework of keratin. It is sometimes detected in the cell nucleus [32] and it influences the formation of the HPV-1 triggered nodules [31]. It also has role in the regulation of cell cycle [32]. E4 protein is expressed at increased levels in cells supporting the viral genome amplification. Its presence in lesions implies its role in the staging of the disease. It has been stated that E4 has potential to disrupt the cellular keratin network and the accumulation of cornified envelope may expedite the release of the virus and/or its transmission [34].

E5 protein is an oncoprotein involved in the transformation by activating EGF receptor and participates in viral DNA replication [31, 32]. This protein also helps the infected cell to avoid recognition by the immune system [35].

E6 and E7 proteins play central role in HPV-dependent malignant transformation. They impair with the cell-cycle control protein [32]. E6 interact with the p53 protein, leading to its proteolytic degradation [36]. E7 protein binds to and inactivates the pRb protein, leading to its degradation, which results in the cell's loss of control over the cell cycle [36, 37]. E6 and E7 oncoproteins may undergo phosphorylation and to

various degrees bind to the target proteins. E6 and E7 protein expression is controlled by E2 protein, a host cellular protein YY1 and pro-inflammatory cytokines [31].

L1 and L2 proteins are capsid proteins of the virus, where L1 is the major protein and L2 is the minor capsid protein [25].

1.2.6 Life Cycle of HPV

HPV is a small DNA virus with a genome of approximately 8000 base pairs [26]. The HPV life cycle strictly follows the differentiation program of the host keratinocyte. It is thought that the HPV virion infects epithelial tissues through micro-abrasions, whereby the virion associates with putative receptors such as alpha integrins and laminins, leading to entry of the virions into basal epithelial cells through clathrin-mediated endocytosis and/or caveolin-mediated endocytosis depending on the type of HPV [38].

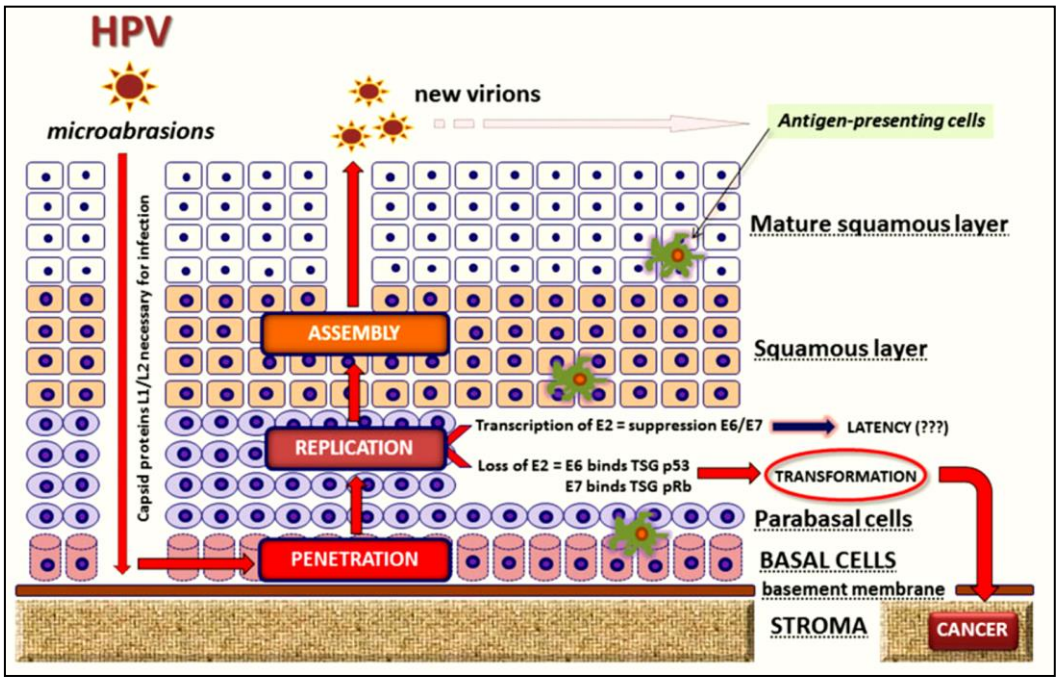

Figure 1.3 HPV enters epithelium through micro-abrasions. Late proteins L1/L2 are needed for penetration into basal cells, and early proteins E1/E2 are necessary for viral DNA replication in parabasal layers. Following assembly in the squamous layers, new virions are released as upper layers are shed. Antigen-presenting cells control expression of the immune microenvironment. Whenever HPV genomes are integrated into the cellular host DNA, disruption of E2 region results in high-level expression of E6 and E7 oncogenes. E6 binds the tumor suppressor gene p53, while E7 binds the tumor suppressor gene pRb. These mechanisms are responsible for the abrogation of cell cycle control, chromosomal alterations, and cell immortalization, eventually causing malignant transformations, and ultimately leading to breakdown of basement membrane and invasion. Source: [39]

Late proteins L1/L2 of the viral capsid are necessary for the penetration of the virus into keratinocytes in the basal layers. Here HPV genomes are established as episomes, and the early proteins E1/E2 are necessary for viral DNA replication in the parabasal layers, where the infected daughter cells begin to differentiate. The assembly of the virions occurs in the squamous layers, through the synthesis of capsid proteins. Subsequently, the new virions are released into the environment as the mature upper layers of the epithelium are shed. The presence/absence of mature antigen-presenting cells determines various degrees of expression of the immune microenvironment, inside which a related immune response is initiated [40]. In low-grade lesions the HPV genomes are found exclusively as episomes, which represent the complete viral gene expression. By contrast in cancer and precancer cells HPV genomes are often integrated into the cellular host DNA. While the transcription of the E2 region leads to suppression of the oncogenes E6 and E7, its disruption results in high-level expression of these two oncogenes. The degradation of p53 and pRb together with telomerase activation are critical steps in cellular transformation; the HPV E5 protein probably acts in the late phase of the viral life cycle to support cell cycle progression. These mechanisms are responsible for the abrogation of cell cycle control, chromosomal alterations, and cell immortalization, eventually causing malignant transformations [41]. Thus cancer ultimately represents an abortive infection in which viral gene expression becomes deregulated and the normal life cycle of the virus cannot be completed [42].

1.2.7 Genotypes of Papillomavirus

According to recent recommendations of the International Committee on Taxonomy of Viruses (ICTV) (http://www.ictvonline.org/virusTaxonomy.asp) this virus belongs to the Papillomaviridae family, which contains 29 genera (30 genera according to ICTV) formed by 189 papillomavirus (PV) types isolated from humans (120 types), non-human mammals, birds and reptiles (64, 3 and 2 types, respectively). The current set of human PVs are contained within five genera, whereas mammalian, avian and reptile PVs are contained within 20, 3 and 1 genera, respectively [43].

For each genus there are biological properties and characteristic genome organization. Some **Alpha-papillomavirus** (types 32, 10, 61, 2, 26, 53, 18, 7, 16, 6, 34, 1, 54) are responsible for mucosal and cutaneous lesions in humans and primates, high- and

low-risk classification based on molecular biological data: high-risk types (pre- and malignant lesions) immortalize human keratinocytes; low-risk types (benign lesions) [44].

Report has been found that 15 HPV types classified as high-risk types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), 3 classified as probable high-risk types (types 26, 53, and 66), and 12 classified as low-risk types (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) [45].

Beta-papillomaviruses (types 5, 9, 49) are responsible for cutaneous lesions in humans. Infections exist in latent form in general population and are activated under conditions of immune suppression.

Gamma-papillomaviruses are responsible for cutaneous lesions in humans, histologically distinguishable by intra-cytoplasmic inclusion bodies specific for the type of species.

Mu-papillomaviruses are responsible for cutaneous lesions.

Nu-papillomaviruses are responsible for benign and malignant cutaneous lesions. This genus is responsible for diseases in humans. Alpha-HPVs infect mucosal tissue, beta-, gamma-, nu- and mu-papillomaviruses infect the cutaneous site [44].

Delta-papillomavirus, Epsilon-papillomavirus, Zeta-papillomavirus, Eta-papillomavirus, Theta-papillomavirus, Iota-papillomavirus, Kappa-papillomavirus, and Lambda-papillomavirus, Xi-papillomavirus Omikron-papillomavirus, and Pi-papillomavirus are responsible for diseases in animals [44].

1.2.8 Diseases caused by HPV

1.2.8.1 Skin warts

All HPV infections can cause warts (verrucae), which are noncancerous skin growths. Infection with these types of HPV causes a rapid growth of cells on the outer layer of the skin. Types of warts include:

Common warts: Some cutaneous HPV types cause common skin warts. Common warts are often found on the hands and feet, but can also occur in other areas, such as the elbows or knees. Common warts have a characteristic cauliflower-like surface and

are typically slightly raised above the surrounding skin. Cutaneous HPV types can cause genital warts but are not associated with the development of cancer [46].

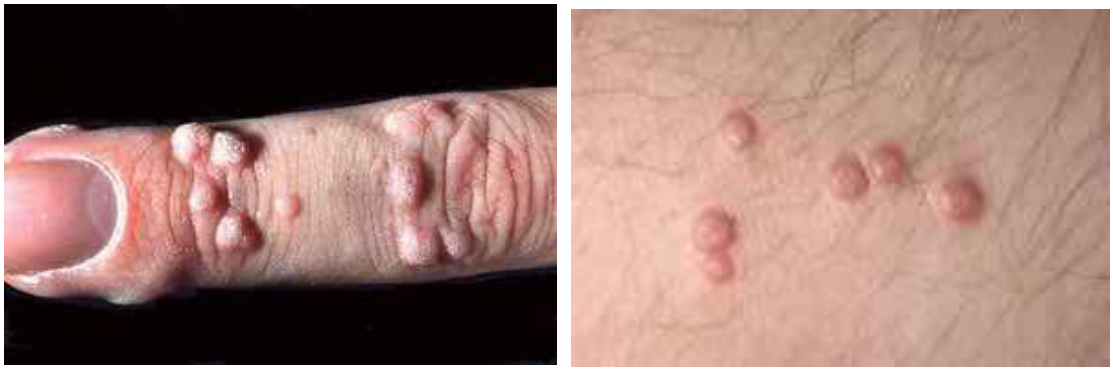

Figure 1.4: Skin warts caused by HPV. Source: [47]

Plantar warts: These are found on the soles of the feet. Plantar warts grow inward, generally causing pain when walking [46].

Periungual warts: These warts form under the fingernail (subungual), around the fingernail or on the cuticle (periungual). They may be more difficult to treat than warts in other locations [48].

Flat warts: Flat warts are most commonly found on the arms, face or forehead. Like common warts, flat warts occur most frequently in children and teens. In people with normal immune function, flat warts are not associated with the development of cancer [47].

Butcher's warts: A rare entity is the presence of widespread warts seen on the hands of butchers. There is no evidence at this time that animal papillomaviruses are responsible. HPV 7 is commonly detected. A combination of maceration and trauma seems to be a predisposing factor [46].

1.2.8.2 Genital warts

Most people who acquire genital wart-associated HPV types clear the infection rapidly without ever developing warts or any other symptoms. People may transmit the virus to others even if they do not display overt symptoms of infection. Genital or anal warts (condylomata acuminata or venereal warts) are the most easily recognized

sign of genital HPV infection. Although a wide variety of HPV types can cause genital warts, types 6 and 11 account for about 90% of all cases [49].

HPV types that tend to cause genital warts are not those that cause cervical cancer. Since an individual can be infected with multiple types of HPV, the presence of warts does not rule out the possibility of high-risk types of the virus also being present. The types of HPV that causes genital warts are usually different from the types that cause warts on other parts of the body, such as the hands or inner thighs [50].

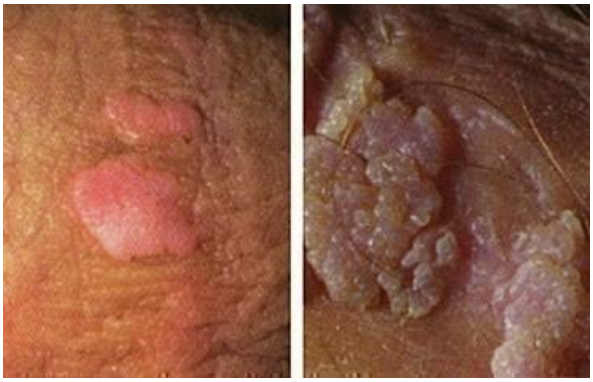

Figure 1.5: Genital warts caused by HPV. Source: [51]

1.2.9 Global statistics of cancer

Cancer with an incidence of approximately 14 million new cases in 2012 is one of the leading causes of morbidity and mortality worldwide [52] and the number of new cases is expected to rise by about 70% over the next 2 decades. Total 8.8 million deaths occurred due to cancer in 2015. Globally, nearly 1 in 6 deaths is due to cancer [19]. Approximately 70% of deaths from cancer occur in low- and middle-income countries. High body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use account for around one-third of deaths from cancer. Tobacco use is responsible for approximately 22% of cancer deaths and recognized as is the most important risk factor for cancer [53]. Cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries [54]. Less than 30% of low-income countries reported the availability of treatment services to public sector compared to more than 90% of high-income countries. The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in

2010 was approximately US$ 1.16 trillion [1]. Only 1 in 5 low- and middle-income countries have the necessary data to drive cancer policy [55].

1.2.10 Cancers caused by HPV

A subset of HPVs is clearly implicated in the development of malignancy in humans. In humans, these HPVs include those found in cervical cancer and anal cancer (especially HPV-16, HPV-18, HPV-31, and HPV-45) as well as those found in cutaneous squamous cell cancers (especially HPV-5 and HPV-8). HPVs are also present in some cases of vulval and vaginal cancer, penile cancer, and oral cancer, especially tonsillar cancer [23]. There is some evidence associating HPV infection with some esophageal cancers, and sensitive HPV DNA detection techniques have identified a variety of HPV types in nonmelanoma skin cancers (NMSCs; basal cell and squamous cell tumors) arising in long-term renal transplant recipients as well as in immunocompetent individual [46]. Some of these cancers caused by HPV are described below:

Cervical cancer: Cervical cancer is cancer arising from the cervix. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body [56]. Early on there are typically no symptoms. Later symptoms may include: abnormal vaginal bleeding, pelvic pain, etc. HPV infection appears to be involved in the development of more than 90% of cases. Strong scientific evidence shows that a long lasting HPV infection is required for cervical cancer to develop. Whether a woman who is infected with HPV will develop cervical cancer depends on a number of factors, including the genotype of the virus. Of the cervical cancers related to HPV, about 70% are caused by two strains, HPV-16 or HPV-18 [5]. There various other risk factors are involved to cause cervical cancer, such as smoking, socio-economic status, using birth control pills for a long time (five or more years), having given birth to three or more children, having several sexual partners and being immunocompromised [2].

Oral cancer: HPV can also be associated with the cause of oral cancer (cancer of the mouth and tongue) and oropharyngeal cancer (cancer of the oropharynx, the middle part of the throat located from the tonsils to the tip of the voice box) in men and women. These HPV-related carcinomas are increasing steadily in both men and

women. Changes in sexual behavior, including an increase in oral sex, may be one reason for the rise [57]. Focal epithelial hyperplasia of the oral cavity (Heck's disease) is caused predominantly by HPV-13 and tends to regress spontaneously [18].

Squamous cell carcinoma of the finger: Squamous cell carcinoma and Bowen's disease (carcinoma in situ) affecting the fingers are special entities. These can be caused by mucosal high-risk HPV, mainly type 16 but also HPV 31, 33, 35, and 52 of species 9 of alpha Papillomaviruses [46].

Head and neck carcinomas: Ninety percent of head and neck carcinomas are squamous cell carcinomas. Recognized risk factors are smoking and alcohol consumption. About 30 % appear to be caused by HPV infection, especially HPV 16 (90 % of HPV-positive carcinomas). The tumors are mainly found in the oropharynx, tonsils, on the base of the tongue and soft palate. HPV transmission via orogenital sexual contact is considered likely [23].

1.2.11 Association of HPV with cervical cancer

Infection of the female genital tract by high-risk HPV types is associated with intraepithelial cervical neoplasia and cancer. The first neoplastic changes noted on light microscopy are termed dysplasia. Approximately 40% to 70% of the mild dysplasias spontaneously regress. Cervical cancer is thought to develop through a continuum of progressive cellular changes, from mild (cervical intraepithelial neoplasia [CIN I]) to moderate neoplasia (CIN II) to severe neoplasia or carcinoma in situ. This sequence of events can occur over 1 to 4 years [25].

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted causes in both men and women worldwide [18]. Ninety-five percent of cervical cancer cases are caused by persistent infections with carcinogenic HPV [58]. Infection with HPV may be latent, subclinical, or clinical. It may take the pathway of low viral load infection without clinical disease, or high viral load infection with clinical disease. In rare occasions, latent or persistent infections may be established by the consistent maintenance of HPV genome in episomal form. This persistence, particularly if caused by certain high-risk HPV types, is a necessary condition for the development of cervical cancer [59].

More than 25 HPV types can infect the genital tract and be associated with cervical dysplasia in some infected women, with no single HPV type accounting for most infections. However, only a subset of the genital-mucosal HPV types is found regularly in cervical cancers. Furthermore, the likelihood of finding certain genital types in more severe dysplasias and in the cancers can be shown to increase rapidly with some types, to remain approximately unchanged with other types, and to decrease with others. These differences, in association with progression to cervical cancer, have led some types to be designated as high risk (such as HPV-16, HPV-18, and HPV-45) [23].

Unlike many genitourinary infections with HPV, is not usually presented with immediate symptoms such as itching, burning, and vaginal discharge [60]. Rather, due to host defense, the majority of HPV infection will not progress to clinical disease or symptoms. The exact mechanism by which HPV infection is cleared by the host immune system is currently unknown. A study shows genital warts developed only in 24.8% of women infected with HPV 6 or 11 [60]. A large, prospective 10-year cohort study of more than 20,000 women enrolled in a health maintenance organization found that approximately 7% in HPV-positive women developed CIN 3 or cancer [61].

Progression of human papillomavirus (HPV) mediated cervical carcinoma is shown in Figure 1.6 below:

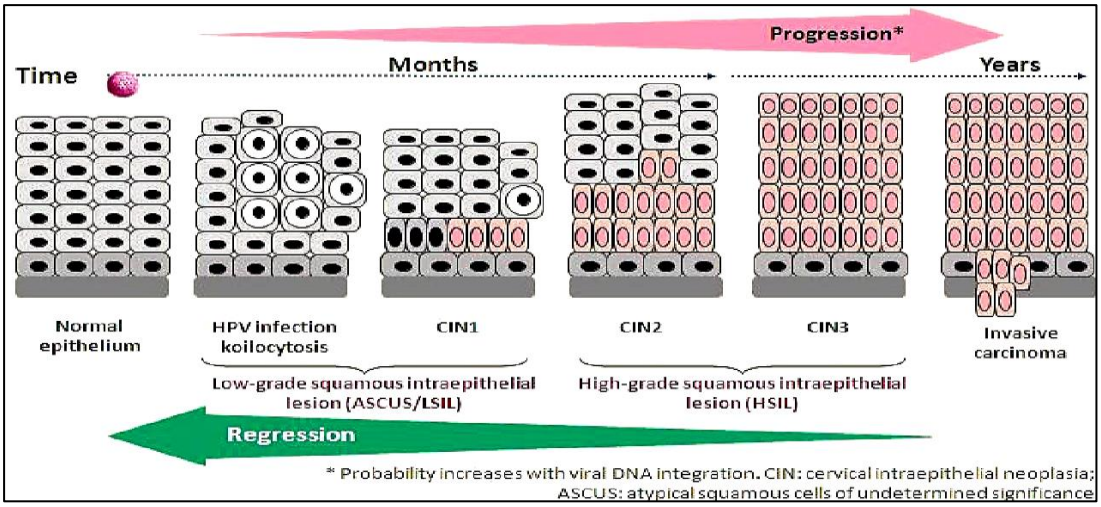

Figure 1.6: Progression of human papillomavirus (HPV)-mediated cervical carcinoma. Source: [25]

1.2.12 Histology of cervix

Endocervix is lined by simple columnar epithelium while ectocervix is lined by stratified squamous epithelium [62]. There is an area called squamocolumnar (SC) junction where squamous and columnar epithelium meets. Transformation zone (also called ectropion) is the area between original SC junction and new SC junction due to regenerative metaplastic response; which is the site of >90% of squamous cell carcinomas and dysplasia.

1.2.12.1 Premalignant condition of the cervix

The premalignant condition of the cervix includes cervical lesions that eventually lead to cervical cancer if untreated. Examples are basal cell hyperplasia, squamous cell metaplasia, leukoplakia, cervical dysplasia and cervical intraepithelial neoplasia (CIN). Various screening tests are available that can detect these pre-cancerous conditions and thus cervical cancer can be prevented.

1.2.12.2 Cervical Intraepithelial Neoplasia

Among these conditions, CIN which is very common is a histological observation where part or whole of the thickness of the cervical epithelium is replaced by varying degree of atypical cells [63].

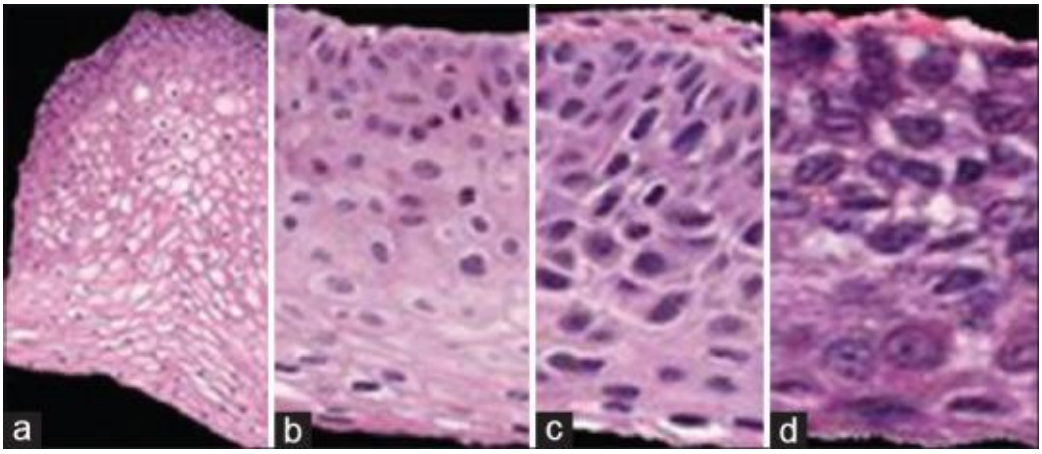

Figure 1.7: Cervical intraepithelial neoplasia grade label examples highlighting the increase of immature atypical cells from epithelium bottom to top with increasing cervical intraepithelial neoplasia severity. (a) Normal, (b) cervical intraepithelial neoplasia 1, (c) cervical intraepithelial neoplasia 2, (d) cervical intraepithelial neoplasia 3. Source: [64].

1.2.12.3 Gross Appearance of cervical cancer

There are three categories of gross appearance of cervical carcinoma:

Exophytic lesions: Most common form and arises on ectocervix. Grows to form large, friable, polypoidal masses that bleed profusely. **Infiltrating lesions:** Presents as stony hard cervix with a minimal or invisible lesion on the cervix. **Ulcerative lesions:** Presents as an ulcer over the cervix, often replacing the whole of the cervix.

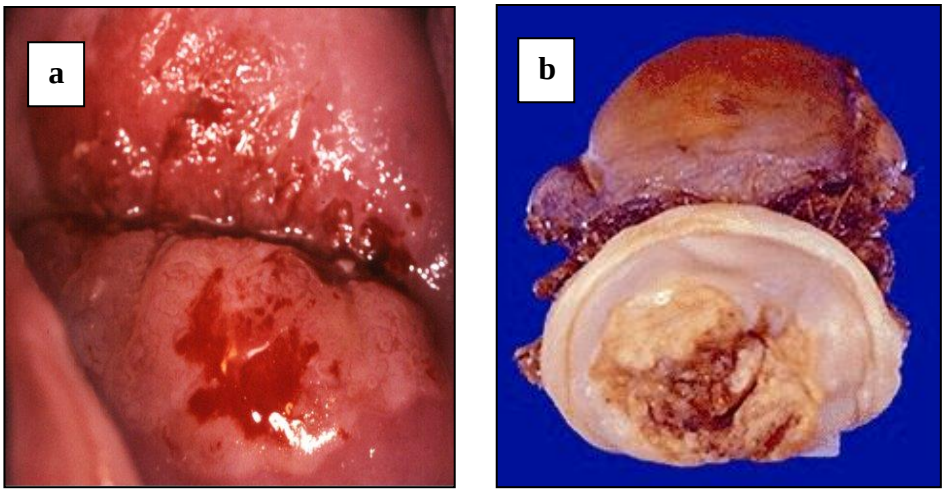

Figure 1.8: Gross Appearance of cervical cancer. a) Exophytic variety [65] and b) Ulcerative type [66].

1.2.12.4 Dissemination and Spread

Like other cancer of epithelial origin, cervical carcinoma metastasizes mainly through lymphatic route. Direct local extension is also a common form of metastasis of cervical cancer. Unusual types of cervical cancer, such as adenosquamous or neuroendocrine tumors disseminates through hematogenous route. Blood spread occurs commonly also in advanced disease.

1.2.12.5 Causes and risk factors for cervical cancer

In recent years, there has been a lot of progress in understanding what happens in cells of the cervix when cancer develops. In addition, several risk factors have been identified that increase the odds that a woman might develop cervical cancer, including the number of sex partners, young age (less than 25 years), lifetime history of sex partners, and the partners' sexual history. Results of epidemiologic studies are less consistent for other risk factors, including age at first intercourse, inconsistent

condom use, number of pregnancies, genetic factors, tobacco smoking, lack of circumcision of male partner, and longtime oral contraceptive use [67].

Besides these, immunosuppression, *Chlamydia* infection, poverty, a diet low in fruits and vegetables, being overweight, intrauterine device use, having multiple full term pregnancies, use of diethyl stibestrol (DES) are also the risk factors of developing cervical cancer [68].

1.2.12.5.1 Age

Age was a significant prognostic factor for cervical cancer [69]. Prognosis is worse with the increase of age. However, cervical cancer tends to occur in midlife. Most cases are found in women younger than 50. Women younger than 20 rarely develops cervical cancer. Around 15% of cases of cervical cancer are found in women over 65 [68].

1.2.12.5.2 Early exposure to sexual intercourse or early marriage

Girls exposing to sexual intercourse in their early teen ages have more chance to develop cervical cancer. It is probably because of vulnerability to HPV infection of young girls.

1.2.12.5.3 Early pregnancy

Women having their first full-term pregnancy below the age of 17 years are in two times higher risk for developing cervical cancer later in life than women who get pregnant after the age of 25 years [70].

1.2.12.5.4 Multiple pregnancies

Multiple births are an important risk factor for cervical cancer. Chance of developing cervical cancer is more with women who have had 3 or more full-term pregnancies. The exact reason behind this remains unknown. One hypothesis is that these women have frequent and more unprotected intercourse to get pregnant, so they may have had more exposure to HPV. Another prediction is that immunosuppression during pregnancy makes women more susceptible to HPV infection.

1.2.12.5.5 Multiple sexual partners

Men or women having multiple sexual partners easily get infected with HPV [63].

1.2.12.5.6 An immunocompromised status

An important risk factor for persistent infections with human papillomaviruses (HPV) and HPV-associated disease is chronic immunosuppression caused by various conditions [71]. Conditions such as Human immunodeficiency virus (HIV) infection, diabetes mellitus, use of chemotherapy, steroid or immunosuppressive drug, chronic kidney disease, malnutrition, protein losing enteropathy, rheumatoid arthritis, multiple myeloma etc. are associated with immunosuppression and people having any of these conditions are prone to HPV infection.

1.2.12.5.7 Smoking

When someone smokes, he and those around them are exposed to many cancer-causing chemicals that affect extra-pulmonary organs as well. Women who smoke are about twice as likely as non-smokers to get cervical cancer. Tobacco by-products have been found in the cervical mucus of women who smoke. Factors that cause a neoplastic change in cervix in smoker women are direct local carcinogenic effect and local immunosuppression [72]. Passive smoking is also associated with increased risk, but to a lesser extent [73].

1.2.12.5.8 Low socioeconomic status

Low socioeconomic status is associated with unhygienic living. Women of these families cannot maintain adequate sanitation during menstruation. This condition makes women of this family vulnerable to HPV infection. They also do not have easy access to adequate health care services, including pap tests. This means they may not get screened or treated for cervical pre-cancerous conditions.

1.2.12.5.9 *Chlamydia* infection

The Chlamydiales order, composed of Gram-negative obligate intracellular bacteria, shares a unique biphasic developmental cycle. The order includes important pathogens of humans and animals [74]. It is a common bacterium that can infect the urogenital system and is transmitted by sexual contact. In women, 70-80% of genital

tract infections with *C. trachomatis* are asymptomatic, but 15-40% ascends to the upper genital tract, which can lead to serious sequelae, including pelvic inflammatory disease, infertility and ectopic pregnancy [75]. Studies have shown that risk of cervical cancer is higher in women having past or current *Chlamydia* infection in cervix or blood [13].

1.2.12.5.10 Dietary habits

A diet deficient in fruits, vegetables, as well as being overweight, increases the risk of cervical cancer [68].

1.2.12.5.11 Long-term use of oral contraceptives

Risk of the cervical cancer is increased with long time taking of oral contraceptives (OCs). Studies have shown that the longer the use of OCs, the greater the risk of cervical cancer [73]. A research, carried out by the WHO's International Agency for Research on Cancer, found that prolonged use of the oral contraceptive pill increased the risk of cervical cancer up to fourfold, but only in women who carry the human papillomavirus (HPV).

1.2.12.5.12 Diethylstilbestrol (DES)

DES increases the risk of adenocarcinoma in the cervix, especially in women whose mothers took DES when pregnant [59].

1.2.12.5.13 Family history

Cervical cancer may run in families. If mother or sister or first-degree female relatives of a woman have cervical cancer, her chance of developing the disease is more. Some researchers predict familial tendency to develop cervical cancer is associated with an inherited condition that makes women vulnerable to HPV infection than others.

1.2.12.6 Signs and symptoms

Abnormal vaginal bleeding which may be irregular or continued in the form of intermenstrual bleeding, contact (post-coital) bleeding or post-menopausal bleeding is a cardinal symptom. The patient may present with offensive vaginal discharge, pelvic pain of varying degree, leg edema and anemia. In advanced disease, metastases to bladder, rectum, pelvic wall, other pelvic organ and distantly to lungs or elsewhere

can occur. In case of bladder involvement, the patient may have complaints of the frequency of micturition, dysuria, hematuria, true urinary incontinence. Features such as diarrhea, rectal pain, bleeding per rectum, rectovaginal fistula indicate rectal involvement [76]. General Symptoms and signs of cervical cancer include cachexia, pallor, loss of appetite, weight loss, fatigue, uremia [77]. However, the early stages of cervical cancer may be completely free of symptoms [3].

1.2.12.7 Diagnosis

Available tests for cervical cancer diagnosis are Pap test, colposcopy, and cervical biopsy. Pap test is a screening test and may be done primarily in suspicious cases. But it is false negative in up to 50% of cases of cervical cancer [78]. Colposcopy is a magnified visual inspection of the cervix aided by using a dilute acetic acid solution to highlight abnormal cells on the surface of the cervix [79]. Colposcopy is recommended if Pap test result is abnormal. A sample of tissue (biopsy) is collected for laboratory testing (histopathology) when an unusual area of cells during the colposcopic examination is seen. Biopsy is the most important investigation in diagnosing cervical cancer [80]. It is a confirmatory test for the diagnosis of cervical cancer which can be done by 3 methods:

- **Punch biopsy:** In this method, small pieces of tissue are taken from the cervix with an instrument called biopsy forceps. Cervix might be stained with a dye to make it easier to see any abnormalities.
- **Cone biopsy:** This surgery uses a scalpel or laser to remove large, cone-shaped pieces of tissue from the cervix.
- **Endocervical curettage (ECC):** During this procedure, cells are removed from the endocervical canal (the area between the uterus and vagina). This is done with a hand-held instrument called a curette.

Once the diagnosis is confirmed, several other investigations including chest x-ray, CT scan of the whole abdomen, ultrasonography of the whole abdomen, colonoscopy, cystoscopy, intravenous pyelography, MRI, liver function test, renal function test are performed to determine the stage of cancer. General physical condition is assessed by CBC, serum electrolyte, ECG etc.

1.2.12.8 Cervical cancer stages

The different stages of cancer describe how far cancer has grown (tumor size) and spread at the time of diagnosis (metastasis). Information from clinical examination and tests is used to determine the size of a tumor, how deeply the tumor has invaded tissues in and around the cervix, and its spread to distant places (metastasis). The FIGO (International Federation of Gynecology and Obstetrics) staging system is most commonly used for cervical cancer [81]. The stages are described using the number 0 and Roman numerals from I to IV.

The AJCC (American Joint Committee on Cancer) TNM staging system classifies cancer on the basis of 3 factors:

- The extent of the main tumor (T)
- Whether cancer has spread to nearby lymph nodes (N)
- Whether cancer has spread (metastasized) to distant parts of the body (M)

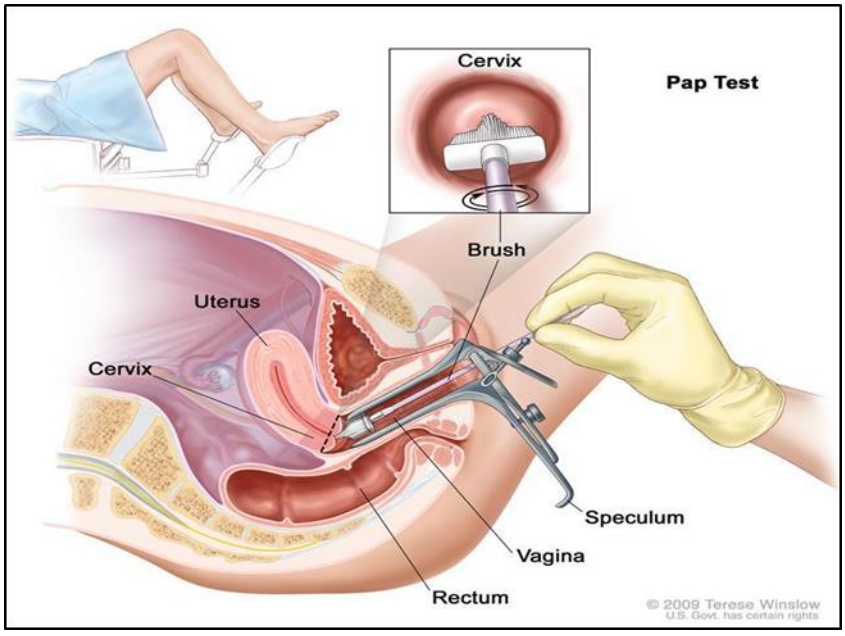

Figure 1.9: Pap test. Source: [68]

Information about the tumor (T), lymph nodes (N), and any cancer spread (M) is then combined to assign cancer an overall stage. This process is called stage grouping. The 5 years survival rates from the time patient presented to clinician of stage 1 is 85%, whereas the rate for stage 4 is 7% (Figure 1.10).

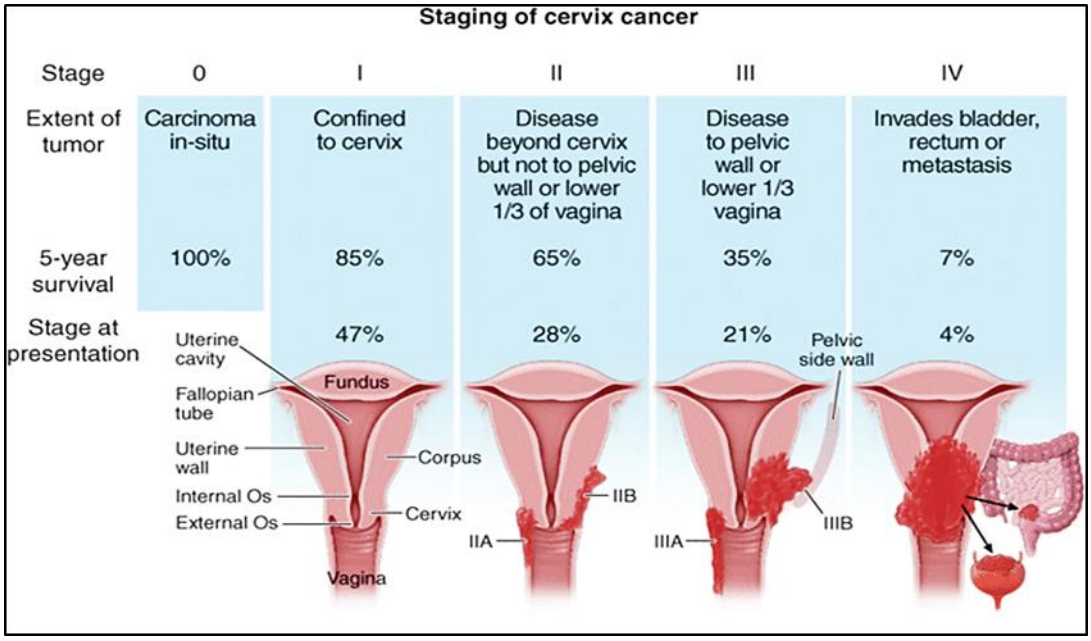

Figure 1.10: Stages of cervical cancer. Source: [82]

1.2.12.9 Treatment option for cervical cancer

The main outcome of any cancer treatment is to preserve life and remove the tumor as quickly as possible. The options for treating cervical cancer depends on different factors including the type and stage of cancer, age of the patient, financial status, preserving reproductive function in case of a younger patient having desire to have children in future. According to type and stages, more than one type of treatment may be needed.

1.2.12.9.1 Surgery

Early stages of cervical cancer are treated by surgery. Surgical options are diathermy, Conization, laser surgery, loop electrosurgical excision procedure (LEEP), trachelectomy, total abdominal hysterectomy, radical hysterectomy, exenteration. But surgery is the treatment of choice and curative only up to stage IB1

1.2.12.9.2 Radiation therapy

Irradiation is a treatment option for advanced cervical cancer. It may be external or internal. External radiation therapy Includes Intensity-modulated radiation therapy (IMRT). External-beam pelvic irradiation is usually given with a combination of intracavitary applications of a dose of 80Gy. Internal radiation therapy uses a

radioactive substance sealed in needles, seeds, wires, or catheters that are placed directly into or near cancer.

1.2.12.9.3 Chemotherapy

As cervical cancer is mostly radiation sensitive, use of chemotherapy is limited to combination with radiotherapy and this type of intervention is called chemoradiotherapy.

1.2.12.9.4 Adjuvant therapy after radical surgery

- **High risk cases:** patients having nodal metastases with positive surgical margins are high risk cases. Treatment option after radical surgery is Adjuvant chemo-radiotherapy with external pelvic radiation along with weekly Cisplatin chemotherapy.
- **Intermediate risk:** cases are defined as an intermediate risk when cervical cancer is presented with the deep invasion of cervical stroma, parametrial extension, lymphovascular space invasion. Only radiation therapy is required after radical surgery.
- **Low risk:** All other patients are low risk cases and no adjuvant therapy is recommended.

1.2.12.9.5 Targeted therapy

They differ from chemotherapy drugs in the sense that they attack only cancer cells without causing damage to normal cells [83]. Monoclonal antibody therapy is a type of targeted therapy that uses antibodies made in the laboratory from a single type of immune system cell. Bevacizumab is a monoclonal antibody that binds to a protein called vascular endothelial growth factor (VEGF) and can be used to treat advanced cervical cancer and recurrent cervical cancer.

Another targeted therapy Pazopanib which act as a kinase inhibitor and inhibits several kinase proteins (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-A, PDGFR-B, FGFR-1, FGFR-3, Kit, Itk, Lck, and c-Fms) that promote growth and proliferation of tumor cells and also neo-angiogenesis. Pazopanib is showing encouraging results in treating cervical cancer [84].

1.2.12.10 Recurrence of cervical cancer

Recurrence rates according to FIGO staging are: stage IB- 10%, stage IIA - 17%, stage IIB- 23%, stages III and IVA - 42% and 74% respectively [85]. Recurrence rate is 1.2% for tumor size less than 2 cm while for tumor size more than 2 cm, the rate is 21% [86]. Cervix, uterus, vagina, parametria, bladder, ureters, rectum, and ovaries are most common sites of pelvic recurrence [87]. Cervical cancer commonly metastasizes to distant sites such as paraaortic lymph nodes (81%), lungs (21%), and supraclavicular lymph nodes (7%) [88]. Clinical features of recurrence depend on the site of metastasis and are characterized by weight loss, inferior limb edema, pelvic limb pain, vaginal bleeding, respiratory symptoms and supraclavicular lymph nodes swelling. Triad of weight loss, leg edema and pelvic pain is characteristic of recurrent disease [89]. Treatment of recurrence is based on recurrence site (local, regional, and/ or distant), disease-free interval symptoms and the type of primary therapy received. FDA approved Bevacizumab plus Cisplatin and Paclitaxel or Topotecan and Paclitaxel in 2014 for treatment of persistent, recurrent, or metastatic cervical cancer [90]. A study describes the overall survival was 17 months with Bevacizumab and chemotherapy, whereas it was only 13.3 months with chemotherapy alone [91].

1.2.12.11 Prevention of cervical cancer

1.2.12.11.1 Screening tests

Cervical cancer screening techniques used in different health care setup are VIA (visual inspection of the cervix), the Papanicolaou test or Pap smear, cervical cytology and HPV DNA testing. Visual inspection of the cervix as an alternative to the cytology-based detection tests is more appropriate to low-resource settings [92]. Inspection is done by two methods, i.e., visual inspection with acetic acid (VIA) and visual inspection with Lugol's iodine (VILI). In the first method (VIA), which is also called as direct visual inspection (DVI), cervix is examined visually using a bright light after application of 3-5% acetic acid using cotton swab. The test is positive if an aceto-white area is observed near to squamocolumnar junction (SCJ). Acetic acid application may lead to reversible coagulation of intercellular proteins which also results in Aceto-whitening. However, the advantage of visual inspection approach is that it gives an immediate result for treatment. The Papanicolaou test or Pap smear which is widely used for the cervical cancer screening has decreased the prevalence

and mortality of cervical tumor [93]. Cervical disease rate decreases up to 80% by screening with Pap test at regular intervals and routine follow up. Unusual results of Pap test suggest precancerous changes and guide the physician to carry out further investigation and start preventive treatment. Another potential screening technique is fluid based cytology that can detect HIV and *Chlamydia* which are also associated with cervical malignancy.

1.2.12.11.2 Human Papillomavirus Vaccines

Two different strategies may be employed for the development of vaccines against HPV based cancers, which are prophylactic and therapeutic vaccines.

1.2.12.11.2.1 Prophylactic Vaccines

Two prophylactic HPV vaccines named Gardasil® and Cervarix® are commercially available among which Gardasil is quadrivalent (HPV 6, 11, 16 and 18) and Cervarix is bivalent (HPV 16 and 18) [94]. HPV major capsid protein, L1 is presented in the form of virus like particles (VLPs) in this prophylactic vaccines. This protein resembles virions, elicit virus-neutralizing antibodies and do not contain oncogenic viral DNA [95]. These two vaccines are recommended for vaccinating young adolescent girl at or before the onset of puberty. It is important for girls to get HPV vaccine before their first sexual contact - because they have not been exposed to HPV. Both of these vaccines are administered by intramuscular route in three doses (0, 1 or 2 and 6 months). Both vaccines have been shown to prevent potentially precancerous lesions of the cervix. They can prevent almost 100% of disease caused by the four types of HPV targeted by the vaccines [96].

1.2.12.11.2.2 Therapeutic vaccine

These VLP based HPV vaccines are obviously not therapeutic but the studies in animals and clinical trial data show the induction of a cell-mediated immune response to L1. It, therefore, requires further investigations to verify the ability of these vaccines in the regression of HPV infection or genital lesions. The therapeutic nature of Cervarix was examined in an interim analysis of a Costa Rican trial [97] and trial indicates that the vaccine did not induce the clearance of genital infections of other HPV types. In the Future II trial, the therapeutic activity of Gardasil was examined. No significant difference in the rate of CIN+ cases was observed in the vaccine vs.

placebo control arm (11.1% vs. 11.9%) [98]. These studies confirm that HPV VLPs based vaccines do not show therapeutic activity. The benefits of prophylactic vaccines in a broad public health perspective will be accomplished if these vaccines can reach to the women where the effective screening programs are absent.

1.2.12.12 Epidemiology

1.2.12.12.1 Worldwide prevalence

Globally, cervical cancer is the fourth most common cancer in women [99]. The incidence of cervical cancer in developing countries has not reduced significantly during the past three decades. Almost nine out of ten (87%) cervical cancer deaths occur in this regions, where it accounts for almost 12% of all female cancers. High prevalence of cervical cancer in the developing countries is associated with poor nutrition and general health status resulting in impaired immune function, ineffective screening programs and other social, behavioral, possibly genetic factors. In the developed world, by contrast, there has been a major decline in cervical-cancer mortality after the introduction of large-scale cytological testing such as Papanicolaou (Pap) smears. HPV screening by pap test in United States has resulted in a 70% reduction in the mortality from cervical cancer during the past 50 years [8].

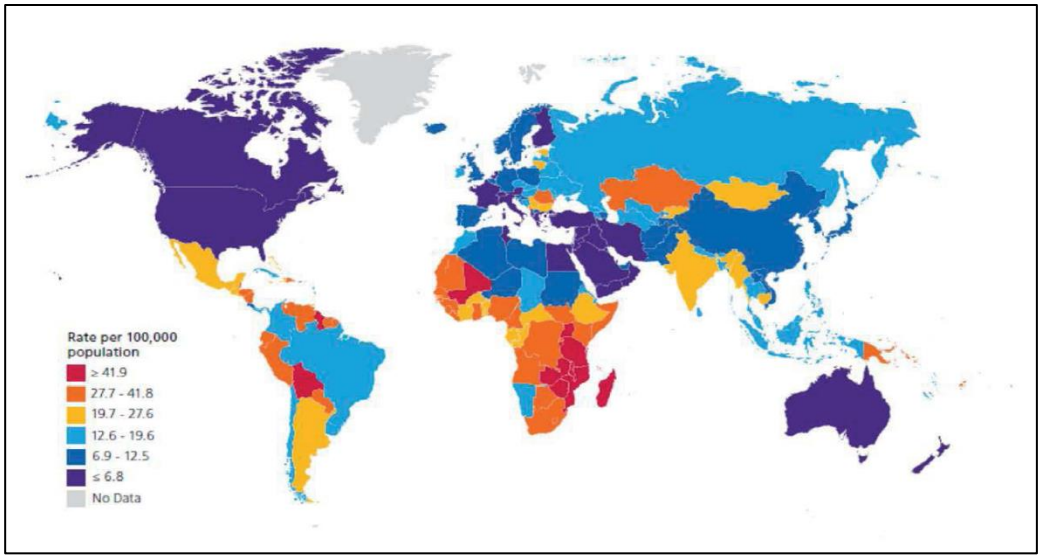

Figure 1.11: International Variation in Uterine Cervix Cancer Incidence Rates, 2012. Per 100,000, age standardized to the World Standard Population. Source: [100]

1.2.12.12.2 Status in Bangladesh

Bangladesh has one of the highest levels of incidence and mortality rates due to cervical cancer among women. The prevalence of cervical cancer in Bangladeshi women is 25–30/100,000 [101]. In a hospital based study of Bangladesh, 96.7% of 120 cervical cancer cases, 83.3% of 36 cases of CIN cases and 4.1% of 120 control women were HPV positive by hybridization [102]. According to the Global Alliance for Vaccines and Immunization (GAVI), Bangladesh stands 11th in the world in cervical cancer fatalities with 17.9 women dying in 100,000 due to the largely sexually transmitted killer disease every year [103].

1.2.12.13 Genes with an effect on cancer progress

Cancer is a genetic disease - that is, cancer is caused by certain changes in genes that control the way our cells function, especially how they grow and divide. Cancer begins when genes in a cell express abnormally due to mutations

Two of the main types of genes that play a role in cancer are oncogenes and tumor suppressor genes. An important difference between oncogenes and tumor suppressor genes is that oncogenes result from the activation of proto-oncogenes, but involvement of tumor suppressor genes lies on their inactivation.

1.2.12.14 Association of mutation in different cancer

Mutations happen often, and the human body is normally able to correct most of them. Depending on where in the gene the change occurs, a mutation may be beneficial, harmful, or make no difference at all. So, one mutation alone is unlikely to lead to cancer. Usually, it takes multiple genetic changes over a lifetime to cause a cancer. This is why cancer occurs more often in older people who have had more opportunities for mutations to accumulate but a mutation in the coding region or in the vicinity of either proto-oncogenes or tumor suppressor genes has a far more pronounced effect on cell cycle regulation and most often leads to cancer.

1.2.12.15 Gene mutation in cancer diagnosis and prognosis

Finding certain mutations in cells can confirm the diagnosis of that cancer. Testing cells for the mutation can also be used after diagnosis to see how the cancer is responding to treatment. Conversely, in some cancers, specific genetic changes can be used to predict which patients are likely to have a better or worse outcome. This can

help guide the intensity of treatment. Both of these are novel ideas which are not widely applied yet. But this line of thinking has a high promise and needs exploring.

Lately, mutation detection is being used for certain cancer types' diagnosis and prognosis. For example, the cells of leukemia patients with chronic myeloid leukemia (CML) contain a mutated gene called *bcr-abl*. In order to be diagnosed with CML, this mutation must be present, so testing for this mutation is used to confirm the diagnosis. Conversely, patients with acute myeloid leukemia (AML) whose leukemia cells have a mutation in the *FLT3* gene have a poorer prognosis than patients whose leukemia cells do not contain that mutation. Doctors may recommend more intense treatment, including stem cell transplant for someone whose leukemia cells have this mutation [104].

1.2.12.15.1 Specific genes of interest and their roles in cervical cancer

Among a large number of genes involved in different cancer, some genes are commonly and frequently associated with the development of common cancers. The protein products of these genes control different signal transduction pathways and these pathways are involved directly or indirectly in various cell cycle and growth regulation mechanisms. Among them, *PIK3CA*, *EGFR*, and *KRAS* are of special interest in this research work.

The involvement of p110α in human cancer has been hypothesized since 1995. Support for this hypothesis came from genetic and functional studies, including the discovery of common activating *PIK3CA* missense mutations in common human tumors [105]. Common human tumors harbor activating *PIK3CA* missense mutations [105]. *PIK3CA* mutations are present in over one-third of breast cancers. Three hotspot mutation positions (GLU542, GLU545, and HIS1047) have been widely reported to date [106]. *PIK3CA* mutation has been found to be oncogenic and is associated with cervical cancers [107].

Somatic mutations in *EGFR* gene results in constant activation of *EGFR* gene leading to uncontrolled cell division [108]. Mutations in *EGFR* gene are commonly found in a number of cancers, including squamous-cell carcinoma of the lung, anal cancers [109], glioblastoma and epithelial tumors of the head and neck [110]. Anticancer therapy directed against EGFR has been developed and called "EGFR inhibitors".

They include gefitinib [111], erlotinib, afatinib, brigatinib, andicotinib. More recently AstraZeneca has developed Osimertinib, a third generation tyrosine kinase inhibitor [112]. Additionally, imaging agents have been developed which identify EGFR-dependent cancers using labeled EGF or anti-EGFR [113].

Somatic *KRAS* mutations are found at high rates in leukemia, colorectal cancer, pancreatic cancer [114] and lung cancer [115]. An activating mutation in the *KRAS* gene is responsible for various malignancies, including lung adenocarcinoma [116], mucinous adenoma, ductal carcinoma of the pancreas and colorectal cancer [117]. *KRAS* upregulates the GLUT1 glucose transporter, thereby contributing to the Warburg effect in cancer cells [118]. However, the impact of *KRAS* mutations is heavily dependent on the order of mutations.

1.2.12.15.2 Relevance of these genes with cervical cancer

An oncogenic mutation which is not always induced by HPV infection affects regulation of cell cycle resulting in excessive growth or tumor of the cervix. Several studies have been done to determine whether specific genes have any significant mutations that can be attributed to cancer. Though research shows that variable oncogenic mutations in different genes rather than in specific one occur in cervical cancer, the PI3K/Akt/mTOR and MAPK/ERK pathways in cervical carcinoma are highly deregulated. A study shows the highest mutation rates were in the genes phosphatidylinositol 3-kinase, catalytic subunit α (*PIK3CA*) (31.3%); Kirsten rat sarcoma viral oncogene homolog (*KRAS*) (8.8%); and *EGFR* (3.8%). *PIK3CA* mutation rates did not differ significantly between adenocarcinomas and squamous cell carcinomas (25% vs 37.5%, respectively; P = .33). In contrast, *KRAS* mutations were identified only in adenocarcinomas (17.5% vs 0%; P = .01), and a novel *EGFR* mutation was detected only in squamous cell carcinomas (0% vs 7.5%; P = .24 [119]. A study found that 14% of cervical cancer cases have *PIK3CA* mutation [120]. Research suggests that HPV induce genomic instability leading to *EGFR* mutation [121].

1.2.12.16 Effects of serum proteins as biomarker

Among a large number of nuclear proteins, high mobility group box-1 (HMGB1), Squamous Cell Carcinoma Antigen (SCCA), Cytokeratin Fragment (CYFRA) 21-1

and carcinoembryonic antigen (CEA) has been found to play an important role in tumor development, growth, and spread. Increased expression of HMGB1 has been reported in several different tumor types, including breast carcinoma [122], colorectal cancer [123], prostate cancer [124], and pancreatic cancer [125], and hepatocellular carcinoma [126]. HMGB1 plays a role in metastasis development, and thus links it to poor prognosis in a variety of cancers [127]. The protein products control different signal transduction pathways and these pathways are involved directly or indirectly in various cell cycle and growth regulation mechanisms. For this reason, serum high mobility group box chromosomal protein 1 (HMGB1), serum squamous cell carcinoma antigen (SCCA), cytokeratin fragment (CYFRA) 21-1, and carcinoembryonic antigen (CEA) are of special interest in this research work. High Mobility Group Box Chromosomal Protein 1 (HMGB1) and its role in cancer.

1.2.12.17 High Mobility Group Box 1 (HMGB1) and its role in cancer

In cancer, overexpression of HMGB1 is associated with each of the hallmarks of cancer including unlimited replicative potential, ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to inhibitors of growth, inflammation, tissue invasion and metastasis. The expression of HMGB1 is regulated by transcription factors including p53, c-Myc, and KLF4 in individual cell types. As a DNA chaperone, HMGB1 participates in DNA replication, recombination, transcription and repair. HMGB1 also been interacts with and enhances the activities of a number of transcription factors implicated in cancer development, including p53, p73, the retinoblastoma protein (RB), members of the Rel/NF-κB family, and nuclear hormone receptors including the estrogen receptor (ER). Cytosolic HMGB1 promotes autophagy and, in particular, mitophagy. HMGB1 is passively released from necrotic cells and is actively secreted by inflammatory cells, binding with high affinity to several receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptors (TLR)-2, TLR-4, TLR-9, and, as a negative signaling molecule, CD24, mediating the response to infection, immunity, autoimmunity, chemotaxis, cell proliferation and tissue regeneration.

1.2.12.18 Squamous Cell Carcinoma Antigen (SCCA) and its role in cancer

Squamous cell carcinoma antigen (SCCA) has been used as a promising aid for the management of squamous cell carcinoma of various sit es. Recently, SCCA gene has been demonstrated at the 18q21.3 loci, and the exon sequence of SCCA gene shows a close homology with inhibitory-type serpins. Actually, SCCA inhibits human chymotrypsin, papain, calpain 1, or cathepsin L. Since serpins are involved in the intercellular adhesion events, it is likely that SCCA takes some part in the malignant behaviors of squamous cancer, e.g. invasion or metastasis. SCCA is also present in the spinous and granular compartments of the mature squamous epithelium. SCCA also appears to play important roles in the stratification or differentiation of the integument.

1.2.12.19 Cytokeratin Fragment (CYFRA) 21-1 and its role in cancer

CYFRA 21-1 is a fragment of cytokeratin 19, a structure protein and part of intermediate filament proteins necessary for stability of epithelial cells. It is thus expressed in a variety of epithelial cells and has already been shown to be a useful biomarker in lung and breast cancer [128-130]. Moreover, CYFRA 21-1 was recently shown to be a prognostic relevant marker for overall survival in metastatic colorectal cancer [129]. However, several research with patients having squamous cell cervical cancer were tested for the presence of cytokeratin 19 (CK 19) fragments to determine the relationship among this parameter, tumor stage, various histopathologic characteristics, and prognosis. For the quantitative determination of CK 19 fragments in serum, the enzyme assay CYFRA 21-1 was used. The aim of this prospective, was to investigate the role of CYFRA 21-1 as serum biomarker in patients with cervical cancer [131].

1.2.12.20 Carcinoembryonic Antigen (CEA) and its role in cancer

Carcinoembryonic antigen (CEA) is a monomer glycoprotein, which is normally found in adult sweat glands, lung epithelia, gastrointestinal epithelia, and various epithelial malignancies. In case of lung and colorectal cancer, pre-therapeutic CEA levels are well established as tumor marker for prediction of time to progression and overall survival; furthermore, CEA kinetics are also known for their important role in the diagnosis of tumor recurrence. In case cervical cancer there are lots research already have been done so far. Carcinoembryonic antigen (CEA) could be an effective

indicator especially as a tumor marker for the evaluation of the response to the treatment of cancers. Therefore, it is necessary to find some sensitive and effective methods for CEA detection.

1.2.12.21 Relevance of these serum proteins with cervical cancer

In a research done on the serum HMBG1 level comparing with SCCA, CYFRA21-1, and CEA level in squamous cell carcinoma of uterine cervix cancer patients [132]. Combined measurements of HMGB1, SCCA and CYFRA21-1 increased the diagnostic sensitivity.

1.3 Rationale of this study

In the context of low socioeconomic condition, we are experiencing an increasing burden of cervical cancer disease and mortality rate is quite high. Studies related to statistics of cervical cancer (incidence, prevalence, death rate), cervical cancer screening test like visual inspection with acetic acid (VIA), PAP test, staging of cervical carcinoma have been carried out in Bangladesh. In Bangladesh, no mutation profiling of the several specific cervical cancer associated genes; HPV genotyping and their associated prevalence; and serological studies in order to identify the potential biomarkers yet to be extensively researched. Without this information, decision of chemotherapy is most cases difficult and become non-specific treatment. Therefore, there is an opportunity for the molecular profiling of cervical carcinoma in our country.

1.4 Aims

Although early cervical cancer can be treated with surgery or radiation, metastatic cervical cancer is incurable and new therapeutic approaches are needed. This project aims at detection, genotyping, and prevalence of HPV in Bangladeshi cervical cancer patients and also in apparently healthy women. The outcome of our result can help the clinicians in prevention and early diagnosis, understanding of prognosis and intervention in treatment modalities of this cancer.

1.5 Specific objectives

The specific objectives of this study are –

1) To detect Human Papillomavirus (HPV) from clinical specimen taken from both regular asymptomatic women and cervical cancer patients by PCR.
2) To determine the prevalence and risk of cervical cancer.
3) Genotyping of HPV by sequencing and type-specific PCR amplification.
4) Correlation between the presence of high-risk HPVs and other risk factors such as age, socio-economic status, age of first intercourse, use of contraceptives, education level, husband's occupation, any complications, number of sexual partners etc.
5) To identify and select mutation rich genomic 'hotspot' regions within genes that are most frequently mutated in cervical carcinoma,
6) To determine protein marker from serum of cervical cancer patient by western blot.
7) To determine the association of *Chlamydia* with HPV

CHAPTER TWO

MATERIALS AND METHOD

2.1 Sample Collection

2.1.1 Test Subjects Selection

As a subject to be used in this project we have selected symptomatic and asymptomatic women aged from 12-45 years in Bangladesh that means who are apparently healthy and also who have developed cervical cancer.

2.1.2 Types of Samples

The genotyping of HPV and other study required cervical swab specimens from apparently healthy, cancerous tissue and serum are collected from women who have already developed cancer. All samples were collected from different region of Bangladesh including Dhaka and outside of Dhaka as per availability. As per rule, all the specimens were collected by experienced personnel - either a trained nurse or a physician.

2.1.2.1 Cervical swab samples

To study the detection and genotyping of HPV cervical swab samples were collected from women who are asymptomatic and apparently healthy. All samples were collected from married, ambulant female individuals who came to seek healthcare facilities at different government hospitals and health complexes in Bangladesh. Samples are taken by trained nurse or a physician. Details are given in Table 2.1.

2.1.2.2 Cervical cancer tissue samples

To detect and genotype HPV and related studies cancer tissue samples were collected from cervical cancer patients from the National Institute of Cancer Research and Hospital (NICHR), Mohakhali, Dhaka and Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka. As per rule, all the specimens were also collected by experienced physician.

2.1.2.3 Serum of cervical cancer patient's samples

For the study of serum antibody production against HPV and serum proteins as a biomarker for cervical cancer detection we have used serum samples collected from women who have already developed cervical cancer. These sera were collected from

cervical cancer patients from the National Institute of Cancer Research and Hospital (NICHR), Mohakhali, Dhaka. As per rule, all the specimens were collected by experienced personnel - either a trained nurse or a physician.

2.1.3 Ethical issues

2.1.3.1 Institutional clearance

All human tissue samples used in this study were used according to the National Health Research Strategy (NHRC) developed by National Research Ethics Committee (NREC) which is under Bangladesh Medical Research Council (BMRC). Institutional ethical clearance was taken from National Institute of Cancer Research and Hospital (NICHR), Mohakhali, Dhaka (Ref. no: NICRH/Ethics/2014/115). Institutional review board (IRB) clearance was taken from Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka (No. BSMMU/2017/151). Ethical Clearance Committee of the University of Dhaka also approved all protocols (Ethical Clearance No. 30/Bio.Fac./2016-2017).

2.1.3.2 Patient's consent

Before collection of the tissue sample, each patient was provided with a consent form (Appendix-III). Upon taking consent, information of the patient was written in a specific data collection sheet (Appendix-IV). All the samples were collected with permission from the patients and the doctors involved. Patients were informed of all terms and conditions.

2.1.3.3 Maintenance of confidentiality

Every effort was made to keep patients' information records private. All personal medical information about the patients and any information obtained from the study of their specimen are preserved with security. Patient's name and identity was used only for data collection and not subject to be disclosed to a third party. All others, including employers, insurance companies, personal physicians, and relatives are refused/prohibited to access the information and to the samples, unless patient provides written permission, or unless we are required by law to do so. Anything that can identify patient is kept in private, protected files. An ID number was assigned to the patient, tissue samples, and information about his/her medical history. Only the investigator(s) named on this consent form was authorized to link the ID number to

patient's name. Any research done on any of the samples was designed in a way that protects the privacy of the patients.

2.1.4 Sample Collection procedure

2.1.4.1 Cervix visualization

During sample collection, the patient was kept in dorsal position. With appropriate illumination, Cusco's speculum was inserted through the vagina to visualize and fix the cervix. The cervix was inspected in this condition and relevant findings were noted.

2.1.4.2 Cervical cancer tissue specimen collection

The biopsy tissue specimen was extracted from cancer patients using a pair of sterile forceps, which was then kept in a screw-capped tube containing 2 mL PBS (Phosphate buffered saline). All tissue specimens were carefully labelled.

2.1.4.3 Cervical swab specimen collection

Cervical swab was taken from regular asymptomatic women's cervix as well as from such women who have already developed cervical cancer.

Cervical Swab was taken from subjects who came for VIA testing by a medical team of BSMMU at different hospitals and health complexes. A brief study questionnaire was provided to each individual to select the suspected patients based on several criteria. These samples were collected from ten different places of Bangladesh in a time period between February 2015 and June 2018. The areas and types of patients those were selected for sampling are listed below in the Table 2.1.

Table 2.1: Selection of females for sampling in different places

Serial No.	Area of Sampling	Type of Females selected for Sampling	No. of Samples
1	Mugda General Hospital, Mugda	Regular asymptomatic	51
2	Tejgaon Health Complex, Tejgaon	„	06
3	Uttarkhan Union Health and Family Welfare Center, Uttarkhan	„	42
4	Upazilla Health Complex, Sorail, B.Baria	„	17
5	National Institute of Cancer, Mohakhali	Patients who have already developed cancer	115
6	Brahmanbaria health complex	Regular asymptomatic	15
7	Noakhali health complex	„	35
8	Anwar Khan Modern Medical College and Hospital	„	08
9	Care Hospital	„	52
10	Rural area of Gazipur	„	22
11	Mohakhali	„	34
12	Khulna health complex	„	30
13	Nachol health complex, Rajshahi	„	33
14	Chandpur health complex	„	35
15	Kurigram health complex	„	30
	Total		**525**

2.1.4.4 Serum sample collection

All patient-derived serum specimens were collected and archived under the protocols approved by the institutional review boards of Dhaka University. Written informed consents were obtained from the patients. Total 74 serum samples were collected in between June 2018 to May 2019 and were performed to analyze the expression of HMGB1, CEA, SCCA and CYFRA21-1, cytokeratin 19 fragments 21-1. All of the cervical cancer patients were clinically staged according to the FIGO staging system. Plasma samples were collected before and after the initiation of treatment and centrifuged at 1500g at 4°C for 10 minutes. The separated plasma was aliquoted and stored at -80°C for future analysis.

2.1.4.5 Sampling instruments

Different types of tools and equipments were used for sample collection. Endocervical swab was taken by cytobrush and kept in PreservCyt® Solution for transportation and storage. Tissue samples from cancer patients were taken with the help of forceps and kept in screw capped vial containing PBS. For collection of serum samples, the blood collection vacuum tubes were used.

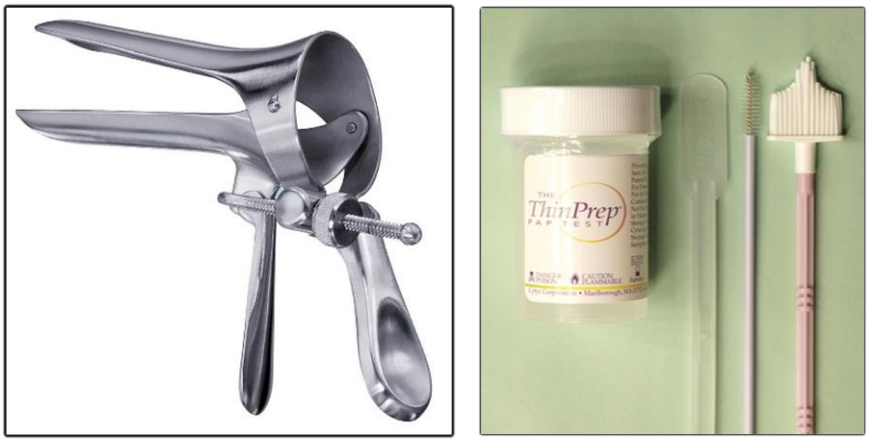

Figure 2.1: Instruments for sample collection. Left: Cusco's vaginal speculum. Right: Thin prep vial containing PreservCyt® Solution, with a cytobrush, cervical spatula and cytobroom that used to collect endocervical swab.

2.1.4.6 Transportation and storage

Samples were transported to the laboratory at the Department of Microbiology, University of Dhaka where all the necessary processing and analyses were carried out. The tissue samples were transported maintaining the cold chain in the form of an insulated ice box. After transport, tissue samples were stored at -20°C until further processing.

2.2 Sample processing

2.2.1 Cervical swab sample processing

The vials containing the cytobrush were kept ion ice for thawing. After about 30 minutes the cytobrush was rinsed in the PreservCyt® solution and was discarded. The half portion of PreservCyt® solution was transferred to a 15 ml falcon tube and was centrifuged at 2000 rpm for 3 min. After centrifugation, the upper layer was decanted

without disturbing the cells at the bottom of the tube. The rest of PreservCyt® solution was again transferred to the falcon tube and the process was repeated. About 0.5 ml samples was kept at the bottom. Then the tube was vortexed at low speed for 15 seconds.

2.2.2 DNA extraction from cervical swab samples

Extraction of total DNA from cervical sample was performed by using QIAamp® DNA Mini Kit (QIAGEN, Germany) followed the instructions provided in the manual. Firstly, 200 µl of processed cervical swab sample was transferred to a 1.5 ml micro centrifuge tube. Twenty microlitres of QIAGEN protease (or Proteinase K) was added to each swab sample. Then the extraction procedure is same for both cervical swab and tissue sample. Five to ten microgram of tRNA was added to 200 µl Buffer AL (lysis buffer) as recommended. This lysis buffer was added to the sample and mixed by pulse-vortexing for 15 seconds. The reaction tube was incubated at 56°C for 10 minutes into a thermo mixture. Then the tube was briefly centrifuged to remove drops from the inside of the lid. Then 200µl ethanol (100%) was added and mixed again by pulse-vortexing for15 seconds. The mixture was applied to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim and centrifuged at 6000 x g (8000 rpm) for 1 min. After centrifugation, the QIAamp Spin Column was placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. Then 500 µl of Buffer AW1(Wash buffer 1) was applied to the QIAamp Spin Column and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Spin Column was again placed in a clean 2 ml collection tube and the tube containing the filtrate was discarded. After that, 500 µl of Buffer AW2 (Wash buffer 2) was applied to the QIAamp Spin Column and centrifuged at full speed (13,000 rpm) for 3 min. After discarding the filtrate containing tube, QIAamp Spin Column was placed in a clean 2 ml collection tube and centrifuged at 13,000 rpm for 1 min to eliminate any chance of possible buffer AW2 carryover. Then the QIAamp Spin Column was placed in a clean 1.5 ml micro-centrifuge tube and 100 µl buffer AE (Elution buffer) was applied to the column. The spin column was incubated at room temperature (15–25°C) for 1 min (for better yield it was effective to keep it for 5-10min) and centrifuged at 6000 x g (8000 rpm) for 1min.For long-term storage of DNA, eluting in buffer AE and store at –20°C.

2.2.3 Cervical cancer tissue sample processing

Tissue sample processing and extraction of total DNA from the cervical sample were performed by using QIAamp® DNA Mini Kit (QIAGEN, Germany) followed the instructions provided in the user's manual.

The initial step was for extracting DNA through homogenization from the tissue sample. A small amount of tissue (<25 mg) was excised with a sterile scalpel and placed in a 1.5 mL centrifuge tube, which contained 180 µL Buffer ATL mixed with 20 µL proteinase K. The tissue was minced with a sterile micropestle to form a relatively even suspension. The tube was then sealed with paraffin film and incubated in a water bath at 56°C until the tissue was completely lysed. During the course of this incubation, the tube was taken out of the water bath and vortexed 2-3 times per hour. The centrifuge tube was centrifuged to remove drops from inside of the lid and the inner walls after the incubation. The incubation period was approximately 3-5 hours.

2.2.4 DNA extraction from cervical cancer tissue samples

Two hundred microliter Buffer AL (lysis buffer) was added to the minced tissue sample and mixed by pulse-vortexing for 15 seconds to yield a homogenous solution. The lysing step has a dual but interrelated purpose: to lyse the non-nucleic acid cell material on one hand and to retain as much nucleic acid as possible. Then the reaction tube was incubated at 70°C for ten minutes in a thermomixer (the shaking helps to dissolve any extant precipitate). Then the tube was briefly centrifuged to remove drops from the inside of the lid. Afterwards, 200µL ethanol (100%) was added to this and mixed again by pulse-vortexing for 15 seconds. The next steps were same as the procedure of extraction from cervical swab samples.

2.2.5 Detection of HPV by PCR amplification and Genotyping

For detection of HPV, conserved L1 region (encodes for major capsid protein) was targeted for PCR (Polymerase Chain Reaction). The MY11/MY09 primer pair-mediated PCR (MY-PCR) and the GP5 /GP6 primer pair-mediated PCR (GP-PCR) are the most frequently used amplification systems for the detection of HPV DNA in clinical samples. Here, in this study, firstly DNA fragment in the L1 region was amplified by using primer pair MY11/MY09 which would give a product of~450 bp

(Table 2.2). Then the product of MY-PCR was amplified by nested PCR using GP5/GP6 primer set which would give a product of~150 bp (Table 2.2).

Table 2.2: Primers used for detection of HPV targeting L1 region

Primers	Sequences (5′⟶3′)	Amplicon size (bp)	Annealing temperature (ºC)	References
MY11 (forward)	GCMCAGGGWCA TAAYAATGGG	450	55	(Sin Hang Lee, 2012)
MY09 (reverse)	CGTCCMARRGGA WACTGATC			
GP5 (forward)	TTTGTTACTGTGG TAGATAC	150	49.2	(de Roda Husman et al., 1995)
GP6 (reverse)	GAAAAATAAACT GTAAATCA			
HPV-16 specific forward (inner)	TACCTACGACATG GGGAGGA	194	55	(Ge et al., 2012)
HPV-16 specific reverse (inner)	GCAATTGCCTGG GATGTTAC			
HPV-18 specific forward (inner)	TGGTGTTTGCTGG CATAATC	339	55	(Ge et al., 2012)
HPV-18 specific reverse (inner)	GCAGCATCCTTTT GACAGGT			

* Key to degenerate nucleotides: M= (A+C), R= (A+G), W= (A+T), Y= (C+T)

2.2.5.1 Preparation of reaction mixture:

Sterile 1.5 ml micro centrifuge tubes (Eppendorf, Germany) were taken and a master mix which include PCR grade water, 10x buffer, $MgCl_2$ of 25 mM, dNTP of 2.5mM, MY 09/11 primers and GP 5/6 primers and Taq polymerase for PCR reaction except the template DNA was prepared. The amount of master mix was prepared according to the number of reactions. The master mix was aliquoted into PCR tubes before adding different extracted DNA samples. After mixing the reaction mixture with the template DNA, the PCR tube containing reaction mixture was capped and centrifuged briefly to spin down the contents. PCR tubes containing master mix and template DNA were placed in thermal cycler (BioRad, USA). The PCR reaction was 94°C for primary denaturation, followed by 35/40 cycles of denaturation at 94°C for 45sec,

annealing temperature 55°C (first round), 49°C (second round) for 45 sec and extension at 72°C for 1:30 minutes and after all the cycle run final extension was kept at 72°C.After this, PCR tubes were stored at -20°C until further analysis. Post - PCR detection of amplified DNA by electrophoretic analysis.

2.2.5.1.1 Preparation of agarose gel

1.5% agarose gel was prepared by dissolving agarose (Sigma, USA) in 1X Tris-borate EDTA (TBE) buffer (Appendix) to give a final concentration of 1.5% agarose and was heated in a microwave oven for about 2.5 - 3 minutes. The melted agarose was allowed to cool to about 50°C. Two microliters of Ethidium Bromide (Et Br) (concentration 0.5 µg/ml) was added in the melted agarose and mixed completely by gentle agitation. The melted agarose with Et Br was poured into gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed and wells were formed. Then the gel was submerged in 1X TBE buffer in a gel electrophoresis unit.

Five microlitres of each PCR product was mixed with 1 µl of 6X gel loading dye. The mixture was slowly loaded into the well using disposable micropipette tips. Marker DNA of known size (100bp ladder) (Bioneer) was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 95 volts for approximately 45 minutes.

2.2.5.1.2 Visualization of the gel

The DNA bands intercalated with Et-Br were observed on a UV transilluminator (Vilber Lourmat, France). Photographs were taken using a gel documentation system (Vilber Lourmat, France) and bands were analyzed.

After this, PCR tubes were stored at -20°C until further analysis. The nested PCR products were resolved in 2.5% agarose gel to analyze the specific band of amplicon. Procedure of loading and running of the sample and staining and visualization of the gel was described in section 2.2.5.2.2

2.2.5.2 Genotyping of HPV

Genotyping of HPV was done to identify that the virus present in the sample was in high risk or low risk group. Two methods were performed to identify genotypes of

HPV. These are -

a) Sequencing of the nested (GP5/GP6 mediated) PCR product
b) Type specific primer set mediated PCR

2.2.5.2.1 Sequencing of the nested (GP5/GP6 mediated) PCR product

After detection of HPV, sequencing of nested PCR (GP5/GP6 primer set-mediated PCR) products (~150 bp) was done using GP5 as forward and GP6 as reverse primer for genotypic analysis of those viruses to determine whether these are high risk HPV or low risk HPV.

2.2.5.2.2 Purification of PCR products

PCR products (150bp) from nested PCR were purified as a prerequisite for DNA sequencing. Purification was performed using Wizard® SV Gel and PCR Clean-Up System (Wisconsin, USA) according to the user manual. Equal volume of membrane binding solution was added to the PCR product. The mixture was transferred to a SV minicolumn which was inserted into a collection tube and incubated at room temperature for 1 minute. It was centrifuged at 16000x g for 1 minute. The flow through was discarded and the minicolumn was reinserted into the collection tube. Then, 700 µl of membrane wash solution was added and centrifuged at 16000xg for 1 minute. Again the flow through was discarded and the minicolumn was reinserted into the collection tube. This step was repeated with 500 µl membrane wash solution and centrifugation at 16000x g for 5 minutes. After that the collection tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuged lid open to allow evaporation of any residual ethanol.

Finally, the minicolumn was transferred to a clean 1.5 ml microcentrifuge tube. Fifty-five micro liters of nuclease free water was added to the minicolumn. After incubating at room temperature for 1 minute it was centrifuged at 16000xg for 1 minute. Then the minicolumn was discarded and purified DNA was stored at -20ºC.

2.2.5.2.3 Measurement of DNA concentration

Concentration of purified PCR products was measured as ng/µl using Nanodrops (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (OD

260 /0D 280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD 260/OD 280 values of 1.8.

2.2.5.2.4 Sequencing reaction

After purification of the PCR products, cycle sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA) according to manufactures instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems®, USA). The basic sequencing protocol is illustrated below:

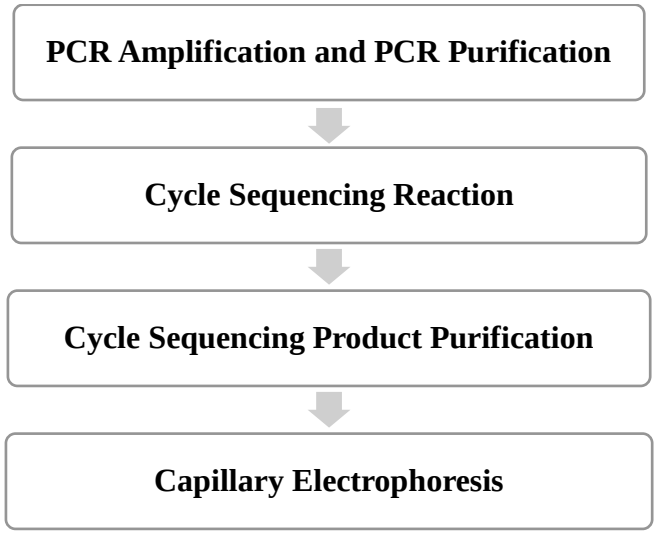

Figure 2.2: Flowchart of basic sequencing protocol

Reaction mixture of cycle sequencing was prepared for either 96-well reaction plates or microcentrifuge tubes to perform cycle sequencing of purified PCR products.

The reaction mixture was added to each tube and mixed well followed by brief spinning. The BigDye® Terminator 3.1 Sequencing Buffer (5X) is supplied at a 5X concentration. For a half of the reaction in the final volume of 20 µl the sequencing buffer was used.

Tubes were place in a thermal cycler and the volume was set in the cycler. The cycle sequencing started with initial denaturation at 96º C for 1 minutes followed by 25 steps of 96º C for 10 seconds, 50º C for 10 seconds and 60º C for 4 minutes. The reactions were hold at 4º C until ready to purify the extension products. The product was then spun down in microcentrifuge. The extension products were purified by Ethanol/EDTA precipitation method. Before starting the precipitation method,

reaction plate was removed from thermal cycler and spun briefly. Then 5 µl EDTA followed by 60 µl of 100 % ethanol was added to each well. The reaction plate was sealed with aluminum tape and mixed by inverting 4 times. After that, reaction plate was incubated at room temperature for 15 minutes. After spinning the plate up to 185 ×g, 60 µl of ethanol was added to each well. The centrifuge machine was set at 4º C and spun at 1600 ×g for 15 minutes. In last step the plate was inverted again and spun at 185 ×g for exactly 1 minute. The plate removed from the centrifuge and sealed with aluminum tape at 4°C.

The sample was then analyzed by ABI Genetic Analyzer (Applied Biosystems®, USA).

2.2.5.2.5 Sequence alignment and identification

Partial sequences, obtained using forward (GP5) and reverse (GP6) primers, were combined to full length sequences (~ 150 bp) via the SeqMan Genome Assembler (DNAstar, USA) and were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the Basic Local Alignment Search Tool (BLAST) [133] to identify different genotypes of HPV.

2.2.5.2.6 Construction of Phylogenetic tree

Phylogenetic tree of the portions of L1 gene sequences of the HPV was constructed using the following bioinformatics software:

a. Reference sequences were download from
    - NCBI: http://www.ncbi.nlm.nih.gov
b. Acquired sequences were aligned, checked and trimmed by using
    - ClustalW [134] and GeneDoc [135]
    - MEGA 5 [136]
c. Phylogenetic tree was constructed by
    - MEGA 5 [136]

Briefly, the multiple sequence alignment of the retrieved reference sequences from NCBI, and representative sample sequences were performed with the ClustalW [134] software. Aligned sequences were exported to the Gene Doc software [135] for sequence trimming and conserved region identification. Refined sequences were

further exported to the Molecular Evolutionary Genetics Analysis (MEGA) [136] software for phylogenetic tree construction using the Neighbor joining algorithm and selecting 1000 bootstrap replication. Further analysis of the genes was carried out using the Distance and Pattern analysis tool in the MEGA software [136].

The phylogenetic tree was inferred using the Neighbor-Joining method [137]. A bootstrap consensus tree was inferred from 1000 replicates [138].

2.2.5.3 Genotyping of HPV using type specific primers

Two sets of type specific primers were used to detect HPV type 16 and type 18. HPV-16 and HPV-18 are high risk HPV genotypes which are the most prevalent etiological agents of cervical cancer so that these two types got the priority for testing, given in Table 2.2.

This was a genotype-specific nested PCR where MY11-MY09 primer amplified product (~450 bp) was used as template. The PCR reaction was performed according to the program described in section 2.2.5.1. After the amplification reaction was completed, PCR tubes were stored at -20ºC until further analysis.

The PCR products were resolved in 2% agarose gel to analyze the specific band of amplicon.

2.3 Antibody detection from serum of cervical cancer patient

Anti-HPV immunoglobulin G (IgG) antibodies of HPV type 6, 11, 16, and 18 (Late protein) detection were performed by enzyme linked immunosorbent assay ELISA using a commercial kit supplied by ALPHA Diagnostic International (USA). The procedure followed as per the instruction provided in the kit. After the last washing step, chromogenic substrate (TMB) was added and colour developed by the enzymatic reaction of HRP with the substrate. Stop solution is added to terminate the reaction, and absorbance at 450 nm is then measured using an ELISA microwell reader. The presence of human IgG antibody in samples is determined relative to anti-HPV calibrators.

As per manufacturer's instructions, cut-off value was defined as the sample's net optical density (OD) by the positive index. The positive index is basically to calculate the net ON mean plus 2 standard deviation value of the control or non-immune samples.

Meanwhile the net OD value was calculated and plotted into the following typical method –

1. Calculate the net **OD mean+2 SD** of the control/Non-immune samples = **Positive Index.**
2. Divide each sample **net OD** by the **positive index**. Values **above 1.0** are a measure of **Positive antibody activity**; **below 1.0** are measures of **negative for antibody**.

A sample would be positive if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel of samples, tested at the same sample dilution. For quantification of antibodies concentration, the calculation also quantifies the positive antibody activity level, assigning a higher value to samples with higher antibody activity and vice versa.

2.3.1 Statistical Analysis

All data were recorded in a database file using Microsoft Excel 2007 [139]. Seroprevalence data was summarized as percentages, and positive antibody titres were presented as geometric means. Differences between percentages were assessed by the Chi square test, whereas differences between geometric mean titres were assessed by Student's t-test of logarithmically transformed values.

2.4 Detection of specific protein in serum as a biomarker

For detection of biomarker protein, specific proteins were first chosen based on a research done on the serum HMBG1 level comparing with SCCA, CYFRA21-1, and CEA level in squamous cell carcinoma of uterine cervix cancer patients [132]. It has been shown that combined measurements of HMGB1, SCCA and CYFRA21-1 increased the diagnostic sensitivity. Thus our target proteins were selected.

2.4.1 SDS PAGE and Western blot

First gel electrophoresis was used to separate proteins from serum of cervical cancer patients based on their size and charge using SDS-PAGE. SDS-PAGE was performed in 12% polyacrylamide and then transferred to nitrocellulose membrane. For sample buffer preparation, a 6x laemelli buffer mixer was made with 50 mM Tris pH 6.8, 20% glycerol, 5% beta-mercaptoethanol.

Bounded proteins on nitrocellulose membrane were analyzed by western blotting. All serum samples were diluted to 1:100 and 1:200. First, the membrane was blocked

using 5% skimmed milk with in TBS buffer. Later, Anti-HMGB1 ab18256, Anti-CEA ab33562, Anti-CYFRA21-1 proteins, cytokeratin 19, fragments 21-1 proteins, ab53119 and Anti-serpin B3 ab 154971 were added gradually to incubate the membrane for 1 hour. Then the membrane was washed using TBST for

Finally, after transfer confirmation, the membrane was blotted using four different antibodies to Serpin B3/SCCA, CEA, HMGB, Cytokeratin 19 fragment protein as a primary antibody. Then a secondary antibody, anti-rabbit IgG HRP conjugated secondary antibody was used. Finally, a substrate [diaminobenzidine (DAB)] was added to identify the antigen-antibody complex.

2.5 Detection of *Chlamydia trachomatis* association with HPV

Chlamydia trachomatis contains a cryptic plasmid with a copy number of 7-10 per bacterial cell. It is sustained in the cell through strong selection process. The plasmid is highly conserved throughout different serovars of C. trachomatis. So we aimed for a region on this plasmid for amplification. We used CC3 (forward) and CC4 (reverse) primers for the amplification of 201 bp region of the plasmid. Sequences and other relevant information for the primer pair are given in Table 2.3**.**

Table 2.3: PCR primer sequences for detecting *Chlamydia trachomatis* plasmid DNA.

Primer used	Orientation	Sequences (5′ ⟶ 3′)	Amplification size	Melting temperature (°C)	Annealing temperature (°C)
CC3	Forward	TAGTAACTGCCA CTTCATCA	201	48	55
CC4	Reverse	TTCCCCTTGTAA TTCGTTCC		50	

2.5.1 Amplification and detection of conserved region

The amplification procedure is described in section 2.2.5.1. Here the CC3 and CC4 primers were added.

2.5.2 Statistical Analysis

After carrying out this diverse array of polymerase chain reactions, statistical analysis was performed on the results.

2.5.2.1 Statistical relationship between cervical cancer and *C. trachomatis*- basic analysis

Table 2.4: Contingency Table to count Odds Ratio (OR)

	Cases	Controls	Total
Exposed	a	b	a+b
Unexposed	c	d	c+d
Total	a+c	b+d	a+b+c+d

OR = (a/c) / (a/d) = (a*d) / (b*c)

Confidence intervals were calculated using the following formulae:

$$95\%\ CI = OR \pm 1.96 * \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}$$

2.5.2.2 Testing for confounder effect by HPV DNA

To test to see whether HPV infection itself is a confounding variable that affects both incidences of cervical cancer as well as C. trachomatis infection, we ran an odds ratio analysis. The groups chosen are presented in Table 2.5. The odds ratio and confidence intervals were calculated as indicated above.

Table 2.5: Study groups chosen for testing confounder effect by HPV DNA

Variables in the Odds Ratio equation	Corresponding sample groups in the study
Number of exposed cases	Samples which test positive for CT among CC samples (CT+, HPV+)
Number of exposed non-cases	Samples which test negative for CT among CC samples (CT-, HPV+)
Number of unexposed cases	Samples which test positive for CT among asymptomatic, HPV (-ve) samples (CT+, CC-, HPV-)
Number of unexposed Non-cases	Samples which test negative for CT among asymptomatic, HPV (-ve) samples (CT-, CC-, HPV-)

2.6 Analysis of mutation in some specific genes

For detection of gene mutation, some specific genes were first chosen. These gene fragments were amplified using designed primer and by PCR reaction.

2.6.1 Primer selection

Information about mutation hotspots of these genes in cervical cancer was gathered from the COSMIC database. For *PIK3CA* and *KRAS* gene, 2 sets of primers were selected as 2 mutation hotspot were reported for these genes in COSMIC. So, total 5 gene segments were amplified by 5 sets of primers. In this dissertation, the gene fragments are named as *PIK3CA*_1, *PIK3CA*_2, *EGFR*, *KRAS*_1, *KRAS*_2 and the primer sets are called as PIK3CA_1 primer, PIK3CA_2 primers, EGFR primer, KRAS_1 primer, KRAS_2 primers respectively. These sequences which represent specific gene fragment of abovementioned genes were applied in primer 3 plus for primer designing. Among the different choices suggested by primer 3 plus, primers having best optimum parameters including primer Tm, GC%, CG Clamp, self-complementarity, 3' self-complementarity, 3' stability, repeat mispriming, pair repeat mispriming, template mispriming, and pair template mispriming were selected. Location of selected primer set in the gene was determined and product size of final

desired gene fragment to be amplified was matched with that of primer 3 plus. Table 2.6 represents the selected primers.

Table 2.6: Primers used for detection of gene mutation.

Target Gene		Primer Sequence	Annealing temp. ºC	Product Size (bp)	GC%
***PIK3CA*_1**	F	AGACAATGAATTAAGGGAAAATGAC	**57**	690	32%
	R	GTTATACCACTCTTCATATAGCTCA			36%
***PIK3CA*_2**	F	AGTGGGGTAAAGGGAATCAAAAGA	**64**	533	41.7%
	R	GCAATTCCTATGCAATCGGTCT			45.5%
EGFR	F	ACGAAGCCTGTGTGTTTGGT	**63**	588	50%
	R	GGCAAAGGGAGTGGAAGGAA			55%
***KRAS*_1**	F	CTTAAGCGTCGATGGAGGAGT	**62**	545	52.4%
	R	ACCCTGACATACTCCCAAGGA			52.%
***KRAS*_2**	F	TGTCCGTCATCTTTGGAGCA	**65**	549	50%
	R	TTTCAATCCCAGCACCACCA			50%

(R= Reverse primer, F=Forward primer. Suffix with an underscore after the gene name indicates primer set number)

2.6.2 PCR amplification of desired gene fragments

2.6.2.1 Preparation of reaction mixture

Five different of PCR reactions were carried out for 5 sets of primer.

Sterile 1.5 mL microcentrifuge tubes were taken and a master mix was prepared which included all the reagents for PCR reaction except the template DNA. The master mix was aliquoted into PCR tubes before adding extracted DNA samples. After adding template DNA to the reaction mixture, the PCR tube was capped and centrifuged briefly for mixing up the contents.

2.6.2.2 PCR condition

PCR condition was optimized for different primers we used. Using prediction of annealing temperature by primer 3plus tool and given melting point by the suppliers, we performed several trials (gradient PCR) to find optimum condition and finally and fixed annealing temperature.

2.6.2.3 Post-PCR detection of amplified DNA of gene fragments by electrophoretic analysis

Amplification of the desired regions was examined by agarose gel electrophoresis. The PCR products were resolved in 1.5% agarose gel to view the specific band of the amplicon.

2.6.2.4 PCR product purification for sequencing

For sequencing, first PCR reactions were carried out to obtain 50 µL products. For this purpose, Pure Link PCR Purification Kit (Invitrogen, USA) was used. The protocol was adopted as per the manual's instruction supplied with the kit with minor modifications.

Purified PCR products were measured as ng/µL using Nanodrop^{TM} spectrophotometer (Thermo Scientific, USA). The ratio between the readings at 260 nm and 280 nm (OD 260 /0D 280) provides an estimate of the purity of the DNA. Pure DNA preparations have OD 260/OD 280 values of 1.8. The tubes containing the purified DNA were then stored at -20°C until sequencing.

2.6.2.5 Sequencing reaction for gene mutation study

The purified DNA fragments were sequenced by a cycle sequencing strategy, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Since fragments were longer than 500 bp, two primers were used to sequence the fragment from both sides.

The protocol adopted was based on the manual supplied with the kit. In this section, the cycle sequencing reaction and clean-up procedures are briefly discussed. After the reaction is run, the products are purified, followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystems, USA).

The reaction mixture of cycle sequencing was prepared for either 96-well reaction plates or microcentrifuge tubes to perform cycle sequencing of purified PCR products. The reaction mixture was prepared as in Table 2.7.

Table 2.7: Preparation of Reaction mixture

Reagent	Quantity
Terminator Ready Reaction Mix	8.0 µL
Template	variable
Primer	3.2pmol
Deionized water	Up to 20µL
Total Volume	**20µL**

2.7 *In silico* analysis

In silico analysis was performed in the following phases:

- Analysis in search of mutations in the target genes
- Genomic and statistical analysis of the identified mutations
- Analysis of the consequences of mutation on protein structure and function

2.7.1 Analysis in search of mutations in the target genes

After gene fragment sequences were obtained, they were carefully analyzed in order to detect possible mutation. For this reason, sequence of each strand was separately compared to the NCBI GenBank [140] database using the NCBI BLASTn [133]. If any inconsistency was found after the alignment, the corresponding chromatogram was scrutinized to determine whether the discrepancy was due to an error in sequencing or by a valid change of base in the sequence. If a valid mutation was found, it was recorded. Mutation location was determined by referring to the GenBank database containing information about the genes of interest. Additionally, types of the found mutations were categorized as well.

2.7.2 Genomic and statistical analysis of the identified mutations

2.7.2.1 Identification of effect of exonic and intronic mutation on gene function

Ensembl Variant Effect Predictor (VEP) [141] tool was applied in search of the effects of the variants of the mutations across Ensembl and RefSeq transcripts

databases using a BED file containing the mutation information**.** This tool outputs the mutation affected genes and transcripts, mutation location in the gene, functional consequence of the mutation and also use of different other integrated tools which include LoFtool [142], SIFT [143], Polyphen-2 [144] and mutation assessor.

2.7.2.2 Prediction of the functional intolerance of genes due to mutation

LoFtool [142] provide a rank of genetic functional intolerance and consequently susceptibility to disease due to the loss of function (LoF) mutation. LoFtool [142] was used in order to find out the intolerance score based on loss-of-function of variants of the mutation. The Smaller the LoF score, the chances are more to become intolerant.

2.7.2.3 Determination of the frequency of exonic mutation

Mutations that were found in the exons were determined. Every particular exonic mutation frequency was also calculated to find out among these three genes which one contains the most exonic mutations in this study.

2.7.3 Analysis of the consequences of mutation on protein structure and function

2.7.3.1 Prediction the effect of non-synonymous mutation on protein function

In order to find out the effects of missense mutations on the protein level, two different scores, SIFT and Polyphen scores, were estimated from the integrated calculation of the VEP tool [141].

SIFT [143] predicts whether an amino acid substitution due to a single nucleotide variation affects the protein function. The higher the SIFT score, the higher chances of being tolerated in the protein structure and function changes. Similarly, PolyPhen-2 [144] tool predicts the possible impact of an amino acid substitution on the structure and function of a human protein. But in this case, lower the Polyphen score, smaller the chance of mutation to have a deleterious effect on the protein function and structure.

MuPro tool [145] was also used and structure related information in order to predict the effect of a mutation on the stability and function of the target protein.

2.7.3.2 Determination of the location of mutated amino acid in the protein

In order to find out in which functional domain of the protein a particular mutant amino acid is located, the MutationMapper tool [146] was used and the missense mutations were mapped and function of the particular domain was searched.

2.7.3.3 Homology modeling and structure comparison between the wild-type and mutant protein

After mutation detection, amino acid sequences corresponding to the nucleotide sequences was carried out to check whether any of the found mutations resulted in a change of amino acid. Amino acid change may lead to protein structure change, so mutant protein was modeled against the wild-type one ***using*** the SWISS-MODEL tool [147]. The generated models have been observed using the UCSF Chimera tool [148] and the models of the mutant and normal proteins were matched to find out the location of the target site of the amino acid and its functional domain.

CHAPTER THREE

RESULTS

Worldwide cervical cancer remains one of the leading causes of cancer-related deaths [149]. With the advancement of the early detection and treatment of pre-invasive disease, invasive cervical cancer rates have steadily decreased over the past several decades. However, treatment of advanced or recurrent cervical cancer is still limited, resulting in poor survival [150]. Although early cervical cancer can be treated with surgery or radiation, new therapeutic approaches are needed for metastatic cervical cancer [151].

In present days, cervical cancer constitutes 25% of all cancer cases in women. It accounts for 13,000 new cases and 8,000 deaths annually. However, cervical cancer is a highly curable disease if detected at early stages [152, 153]. But in our country, >80% patients are diagnosed with this preventable cancer in clinically advanced, inoperable stages [154]. To reduce the mortality rate of cervical cancer Bangladesh Government has introduced a regular screening program named VIA test to detect it in a pre-cancerous stage. The project is jointly conceived and supported by BSMMU and UNFPA, Bangladesh fund. The VIA based screening program is chosen because of its affordability, and it is easy to perform method and achievement of instant results [155].

Oncogenic mutations in human genes which are not induced by HPV infection play a major role in the formation and progression of cervical cancer. Whereas some researches on cervical carcinoma have been carried out in Bangladesh which are on epidemiology, screening tests, staging of cervical carcinoma, HPV vaccination etc.; research related to genetic marker or gene mutation in cervical cancer has not been done yet in Bangladesh. In this study, genetic mutations in specific host genes were detected. Sera samples were collected and analyzed by ELISA to detect antibody against genotype 6,11,16,18 and 43.

3.1 Cervical cancer screening by VIA test and demographic data of samples

For this study, cervical swab specimens were taken by trained nurse from apparently healthy, ambulant, married women aged 18 years or above who came for VIA testing at different hospitals and health complexes of Bangladesh. A brief questionnaire was used to select the subjects based on several criteria.

These samples were collected in a time period between November 2017 and April 2019. Number of asymptomatic, apparently healthy patients selected for sampling is given in table 3.1 with demographic data. As it is revealed in the table that majority of the patients in this study belonged to the age group 15 to 30 years; which is considered as child bearing age and most women likely get contaminated within this age group.

After counseling and informed consent, a speculum examination was performed for direct visualization of the cervix and vagina. Freshly prepared 5% dilute acetic acid was applied to the cervix. VIA test was considered positive when a definite, well-defined aceto whitening was observed in the transformation zone in close proximity with the squamo-columnar junction (SCJ) or when a growth turned aceto white. Only 4 VIA positive cases were found out of 116 patients. This is because the subjects were almost asymptomatic and did not have any major complication. However, a good number of women complained about pain in lower abdomen and foul smelling per-vaginal discharge. The sexual behavior of the subjects was also investigated. All of the participants had single sexual partner.

Table 3.1: Number VIA positive cases in asymptomatic, apparently healthy patients selected for sampling based on several criteria

Criteria for patient selection		Number of patients
Age	Below 20years	5
	20-29 years	7
	30-39 years	45
	40-49 years	55
	50-59 years	9
	Above 60 years	3

Criteria for patient selection		Number of patients
Any complications	Pain in intercourse	0
	Lower abdominal pain	22
	Bleeding during period	5
	Backache	2
Economic status	Poor	14
	Middle	20
	Higher	18
Contraception	No	10
	OCP	28
	Injection	5
	Barrier	2
Sexual Behavior	Single Partner	121
	Multiple Partner	0

3.2 Molecular detection of HPV DNA in samples by Polymerase Chain Reaction (PCR)

A total of 525 samples collected from 525 subjects were analyzed for the detection of HPV DNA. Conserved L1 region (encodes for major capsid protein) of the HPV genome was targeted for PCR (Polymerase Chain Reaction).

3.2.1 PCR amplification with MY11/MY09 primer pair

All the samples were analyzed by PCR amplification with MY11/MY09 primer pair (MY-PCR). Here, MY11 and MY09 were the forward and reverse primer respectively. Few samples showed bands in specific position (~450 bp) upon agarose gel electrophoresis detecting the presence of HPV.

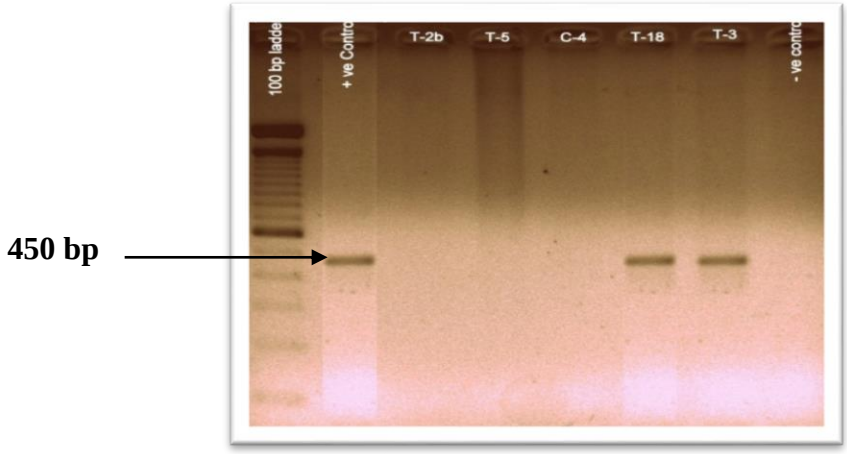

Figure 3.1: Agarose gel electrophoresis of PCR specific amplicon (450 bp) using MY11/MY09 primer pair. Lane 1 = 100 bp ladder (Bioneer, USA).

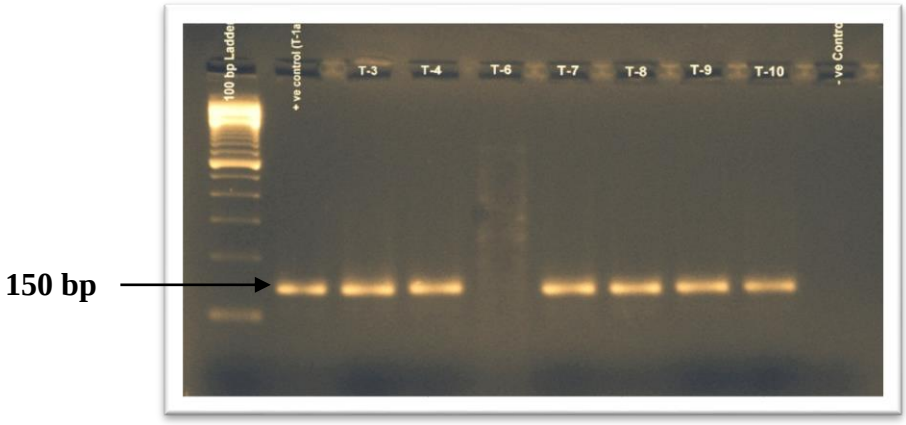

Figure 3.2: Agarose gel electrophoresis of PCR specific amplicon (150 bp) using GP5/GP6 primer pair. Lane T_3-T_4 and T_{-7} to T_{-10} are positive, T_{-6} is negative.

A nested PCR was performed using GP5/GP6 as inner primers to increase the sensitivity of HPV detection. This GP5/GP6 primer pair-mediated PCR gives a product of approximately 150 bp. All the samples were amplified by GP5/GP6 primer pair. Samples those showed specific sized amplicon (~150 bp) upon agarose gel electrophoresis are positive and indicating the presence of HPV.

Number of total samples those were analyzed by PCR amplification is summarized in the Table 3.2. While detecting the HPV by nested PCR amplification, it has been found that approximately 29.5% samples of cervical swab of healthy women were HPV positive, whereas approximately 98.26% cervical tissue samples were positive for the women who already developed cervical cancer.

Table 3.2: Detection of HPV by PCR amplification using both MY11/MY09 and GP5/GP6 primer pairs

Samples taken from healthy individuals		Tissue samples from cancer patients		Total number of samples
HPV Positive	**HPV Negative**	**HPV Positive**	**HPV Negative**	
121 (29.5%)	289 (70.5%)	113 (98.26%)	2 (1.74%)	525
410		115		

3.2.2 Genotyping of Human Papillomavirus (HPV)

The HPVs were genotyped to identify that whether the virus is of high- or low-risk group. In this study, genotyping of the positive samples were done by using two different methods. These are:

(a) Genotyping by using type specific primers (HPV-16 and HPV-18 specific primers).
(b) Genotyping by sequence analysis.

3.2.3 Type specific PCR based genotyping

Type specific PCR based genotyping involved the use of type specific primers that only amplify a single type of HPV specifically. Here, two sets of type-specific primers, HPV-16 and HPV-18 specific primer pairs were used to identify these two high-risk HPV genotypes. This was a nested PCR where MY11-MY09 amplified product (~ 450 bp) was used as template. Genotype-specific nested PCR with type specific primers was done as multiplex PCR where two pairs of primers (HPV-16 and HPV-18 specific primers) were used in a single reaction. Presence of HPV-16 and

HPV-18 showed 194 bp and 339 bp amplicons upon agarose gel electrophoresis, respectively. Presence of single band in a lane indicates a single type of HPV in the sample.

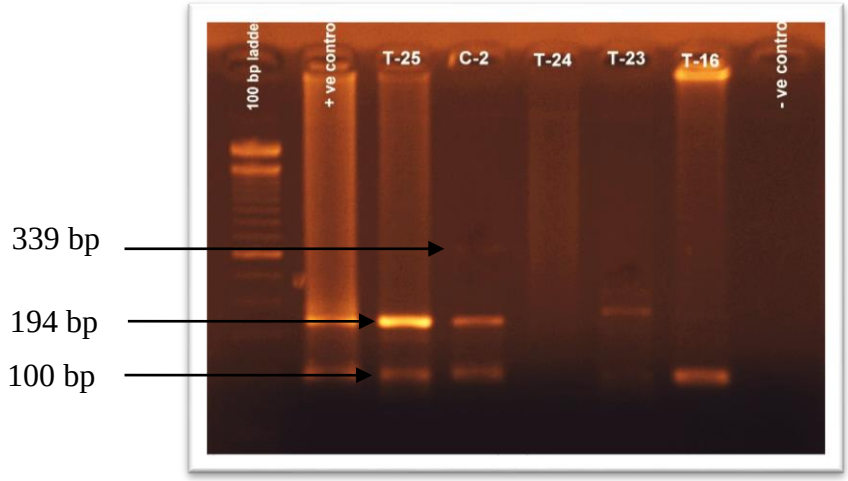

Figure 3.3: Agarose gel electrophoresis of nested multiplex type specific PCR using HPV16 and HPV-18 specific primer pairs. Positive control is showing bands in both positions. T-25, C-2 and T-23 show amplicons in both 339 bp and 194 bp positions indicating that both HPV-16 and HPV-18 are present in the same sample simultaneously; whereas T-16 shows amplicon in 194 bp position indicating HPV-16. Sample no. T-24 is not showing any band, i.e. this sample does not have HPV-16 or 18.

After being detected by MY/GP PCR, 35 HPV-positive samples were tested with this Nested Multiplex Type Specific PCR to determine the genotype of HPV present in the sample.

The findings of this type specific multiplex PCR is given below where is can be seen that among all the HPV positive samples HPV 16 and HPV 18 types are the most predominant either singly or in combination. Approximately 53.7% and 25.61% cervical swab samples were found HPV16 and HPV18 positive, respectively; whereas 46.96% and 38.26% cervical cancer tissue samples were found HPV16 and HPV18 positive, respectively. But in cases of combinatorial infections, combination of HPV16 and HPV18 is found to be the most prevalent one. All the details are given in the table 3.3.

Table 3.3 Genotyping by type specific PCR

HPV types	No. of positive swab samples taken from healthy individuals	No. of positive tissue samples taken from cancer patients	Total
HPV-16	65	54	119
HPV-18	31	14	45
HPV-16 + HPV-18	20	44	62
HPV-16 + HPV-69*	02	0	02
HPV-6*	01	0	01
HPV-62*	01	0	01
HPV-90*	01	0	01
HPV-70*	0	01	01
HPV-31*	0	01	01
HPV-33* + HPV-6*	0	01	01
Total	**121**	**115**	**236**

* Types confirmed from the sequencing results

After the assessment of detection of HPV genotypes here, further overall distribution of HPV infected individuals in the different age ranges has been perceived. At this point an idea could be generated which age groups of women are more susceptible to get infected with HPV. As mentioned earlier, women from very early age like below 20 to age up to 65 have taken into consideration which later on evolved that amongst the 410 women, middle aged women especially from 30 to 50 years are very prone to get infected with HPV. In the table 3.4 shown below, given all the data for the presence of HPV infection both single and multiple types on different age group of women.

Table 3.4: Overall distribution of HPV infected individuals in the different age ranges.

Infection	<20	20-29	30-39	40-49	50-59	>60
HPV -ve	**3**	**33**	**205**	**38**	**8**	**3**
HPV +ve	**5**	**7**	**45**	**55**	**9**	**0**
Single HPV infection						
Low	**0**	**0**	**3**	**3**	**0**	**0**
High	**0**	**17**	**24**	**24**	**3**	**0**
Total	**0**	**17**	**29**	**27**	**3**	**0**
Multiple HPV infection						
Low-Low	**0**	**0**	**0**	**1**	**0**	**0**
High-Low	**0**	**0**	**2**	**5**	**0**	**0**
High-High	**0**	**0**	**20**	**15**	**2**	**0**
Total	**0**	**0**	**22**	**21**	**2**	**0**

3.2.4 Genotyping by sequence analyses

Sequencing of nested PCR (GP5/GP6 primer pair-mediated PCR) products (~150 bp) was done using GP5 as forward primer and GP6 as reverse primer for genotypic analysis. These amplicons (~150 bp) were selected and purified for sequencing analysis.

After sequencing reaction, partial sequences obtained using forward (GP5) and reverse (GP6) primers were combined to full length sequences (~150 bp) and were compared to the Gen Bank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) [133]. As previously mentioned, of samples, 121 were found to be positive after nested PCR. Among the 121 positive samples, 63 unknown type samples were selected for sequencing. The sequencing data shows that all of them showed 97-99% identity to HPV strains as found by BLAST search. Of

the 63 samples, 47 samples belonged to the genotype 16, 11 belonged to the genotype 18; and rests 3 belonged to the genotype 6, 62 and 90; one in each type. Therefore, it is clear that most prevalent genotype circulation in our country is type 16, which is considered as high-risk genotype phylogenetic analysis of the sequence data of the samples.

3.2.5 Phylogenetic analysis of the sequence data

Phylogenetic analysis of the sequence data of the samples was done. Phylogenetic tree was constructed in MEGA6 software using Neighbor-Joining algorithm and 1000 bootstrap replicates to deduce close phylogenetic relationship between the different genotypes of HPV.

Figure 3.4 shows that a phylogenetic tree constructed with 15 HPV samples those are detected by PCR and sequencing. Eleven samples (Sample IDs- 02, 04, 05, 06, 07, 08, 09, 20, 21, 28, I16) were grouped with the HPV genotype 18 of reference sequences. The other four samples having sample ID-16, 27, 24 and I14 were clustered with four different genotypes, HPV type 62, type 16, type 6 and type 90 respectively. Outgroup from the root is shown using reference sequence of L1 region of Bovine papillomavirus type 2.

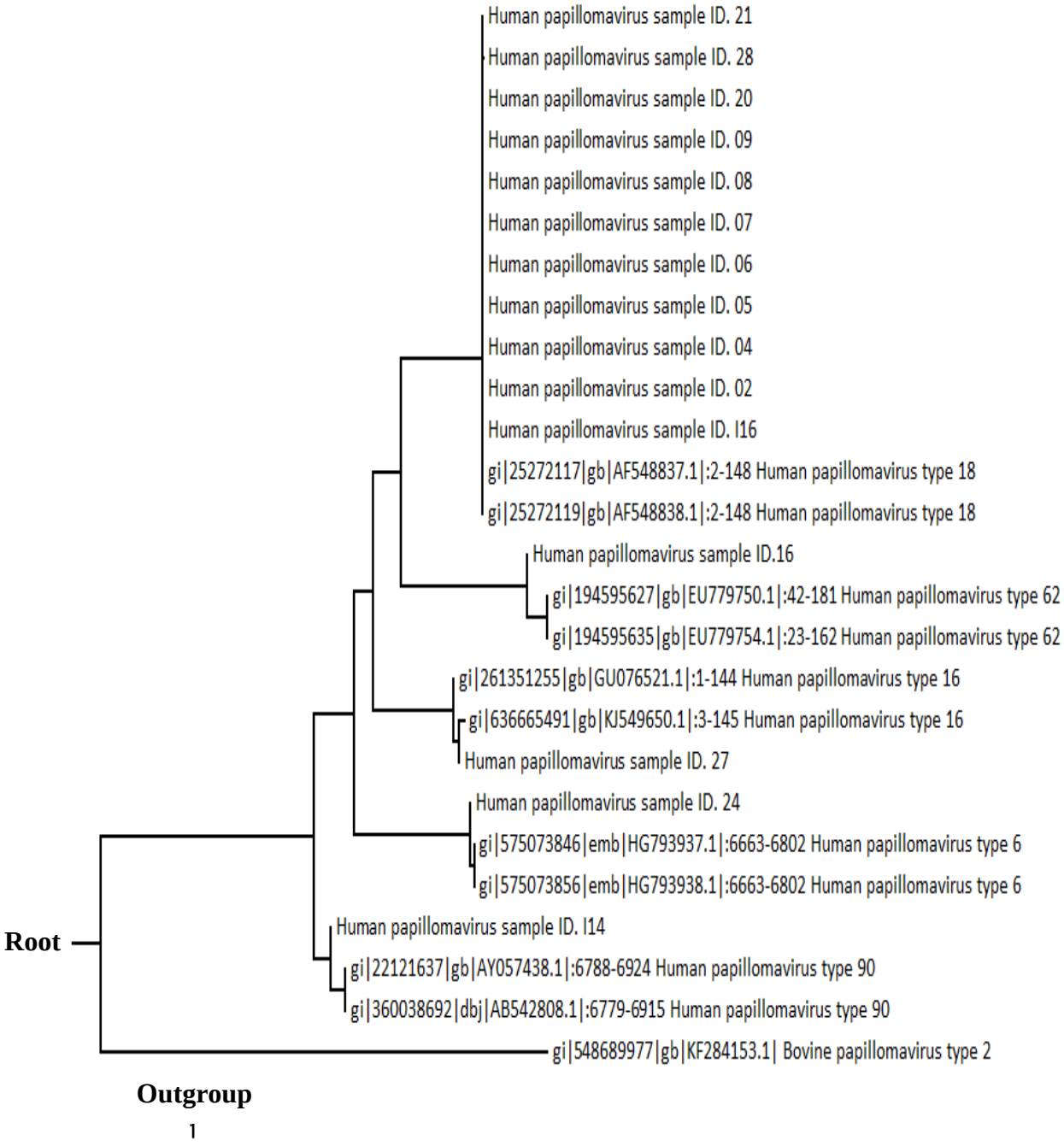

Figure 3.4: Phylogenetic tree constructed using 150 bp nucleotide sequence of L1 region of HPV.

3.3 Correlation between prevalence of HPV with demographical parameters along with statistical analysis

We hypothesized that there might be a significance correlation between prevalence of HPV with different types of demographical parameters. As a matter of fact, to find out some correlations of these parameters with the prevalence of HPV.

3.3.1 Correlation between prevalence of HPV with different age group of women

Genotyping reveals the type of HPV which can detect High-risk or Low-risk HPV present in the cervical samples. As specimens were collected from different age groups, presence of High-risk HPV (mainly HPV-16 and HPV-18) and the age of patients were evaluated to find out any correlation. The evaluation is as follows:

It has been found that high-risk HPVs are present predominantly in the age group 31-45 years. A high number of high-risk HPVs are also shown in the age group 20-30 years in asymptomatic individuals. Broadly it can be concluded that high-risk HPVs (HPV-16 and HPV-18) are prevalent in the age between 15-45 years which is statistically significant (p value < 0.05), It can be interpreted that women get HPV in early ages (after initiation of sexual life) and it develops cervical cancer after 15-20 years later.

3.3.2 Correlation between prevalence of HPV with different socio-economic status

Presence of high-risk HPV in the patients was also evaluated with the socio-economic status of the patients. This is because socio-economic status correlates with the maintenance of hygiene. Unhygienic condition may increase the chance of infection. Evaluation of correlation between high-risk HPV and socio-economic status of the patients are given below:

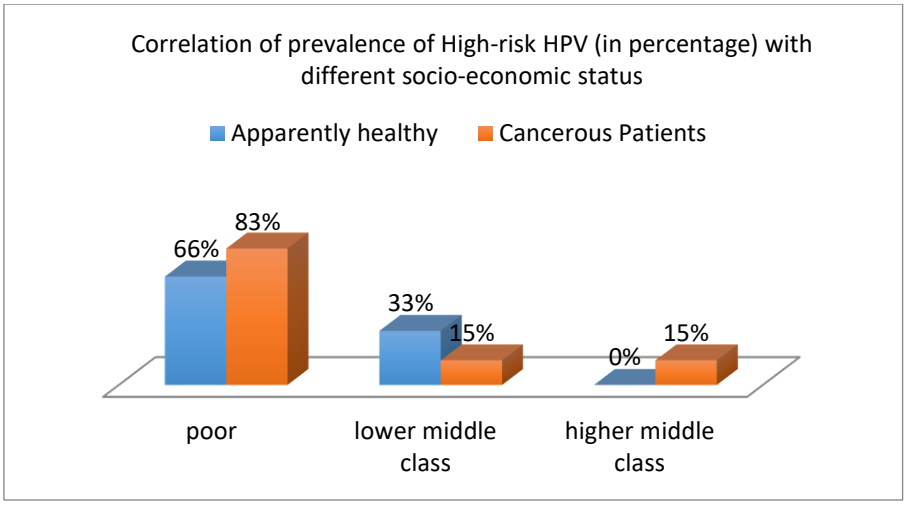

Figure 3.5: Prevalence of High-risk HPV (in percentage) with different socio-economic status (*p* value = 0.0914).

It can be seen in the above figure 3.5, that females with lower socio-economic status are more prone to get infected by HPV and they develop cancer in greater number than other socio-economic class. As the economic status increases, chance of infection decreases. In this study, 66% (apparently healthy) and 83% (cancerous patients) of the samples were coming from females with poor socio-economic status.

3.3.3 Correlation between prevalence of HPV with the contraceptives used by the women

Amongst different factors contraceptive used by the women was one of the major consideration, where it has been seen that the highest number of women have been found HPV positive who used oral contraceptives during their sexual life (53.8%). However, women who never used any contraceptives was also found HPV positive in a considerable number.

Table 3.5: Prevalence of HPV with contraceptives used by the women

Contraceptive used by the women	No of HPV positive patient	No of HPV negative patient
Condom	1	9
Injectables	5	18
OCP	28	83
Withdrawal method	6	10

Contraceptive used by the women	No of HPV positive patient	No of HPV negative patient
Intra Uterin Device	1	3
Tubectomy	2	1
Vacetomy	0	1
OCP and Condom	0	1
No contraceptives	10	63

However, when these data were used for a statistical analysis and graphical representation, it showed not of a major significance (Figure 3.6).

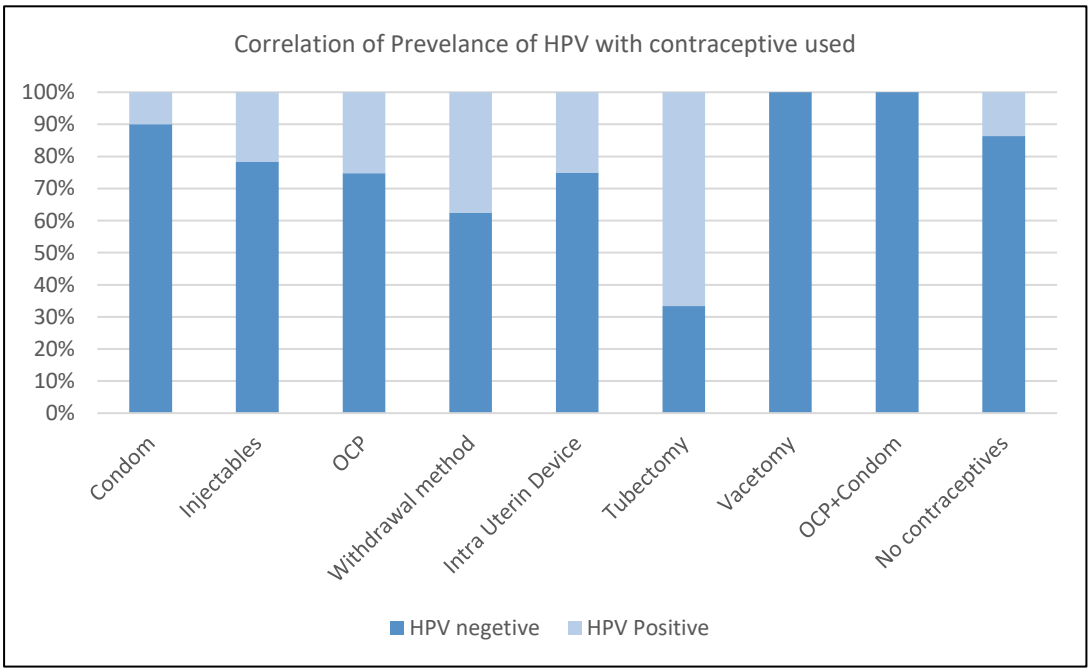

Figure 3.6: HPV prevalence with contraceptive used (*p* value = 0.142).

3.3.4 Correlation between prevalence of HPV with education level of the women

For the study of correlation between prevalence of HPV with the education level of women it has been observed that education has an impact on this HPV infection. Due to lack of knowledge women are unaware of the preventive measures of HPV infection.

Table 3.6: Prevalence of HPV with the education level of the women

Education level	HPV positive patients	HPV negative patients
Higher	4	24
Secondary	13	53
Primary	23	66
Illiterate	10	14
B.A.	1	1
MBA	0	1

Furthermore, presence of high-risk HPV in the women that evaluated with the education level of the women was found to be significant (p value <0.05), the graphical representation revealing the fact below in figure 3.7.

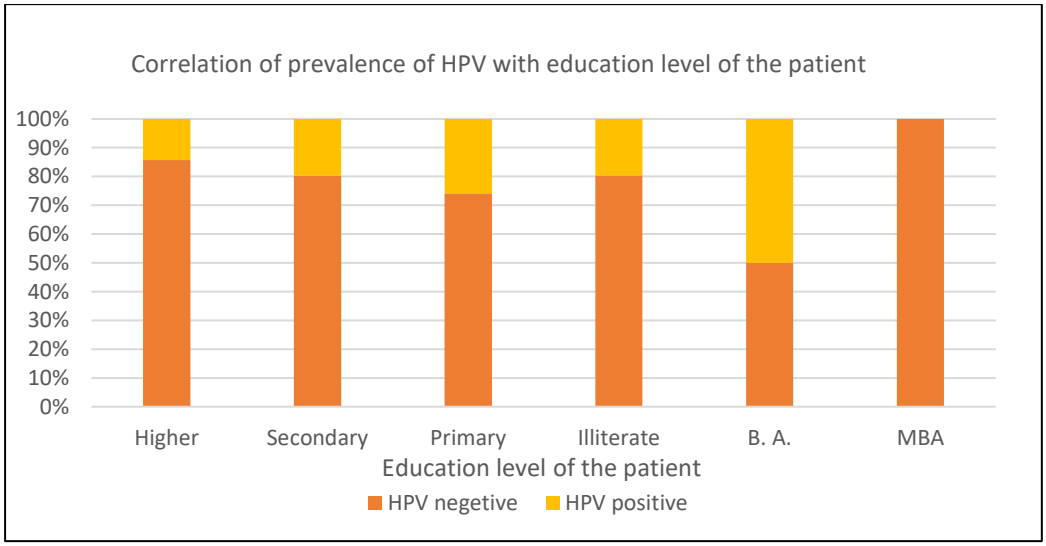

Figure 3.7: HPV prevalence with education level of the patient (p value = 0.046).

3.3.5 Correlation between prevalence of HPV with husband's occupation of the women

Presence of high-risk HPV in the individuals was also evaluated with the socio-economic status of the patients. This is because socio-economic status correlates with the maintenance of hygiene. Unhygienic condition may increase the chance of

infection. Evaluation of correlation between high-risk HPV and socio-economic status of the patients are given below:

Table 3.7: Prevalence of HPV with husband's occupation of the women

Husbands Occupations	HPV negative patient	HPV Positive patient
Self Employed	39	20
Service holder	52	10
Labor	24	5
Farmer	44	8
Teacher	4	0
Retired	1	1
Tailor	1	1
Foreign	2	1
Rickshaw Puller	3	0
Driver	5	1
Jobless	6	1
Disable	2	0
Divorced	1	1
Conductor	1	1
Died	1	2

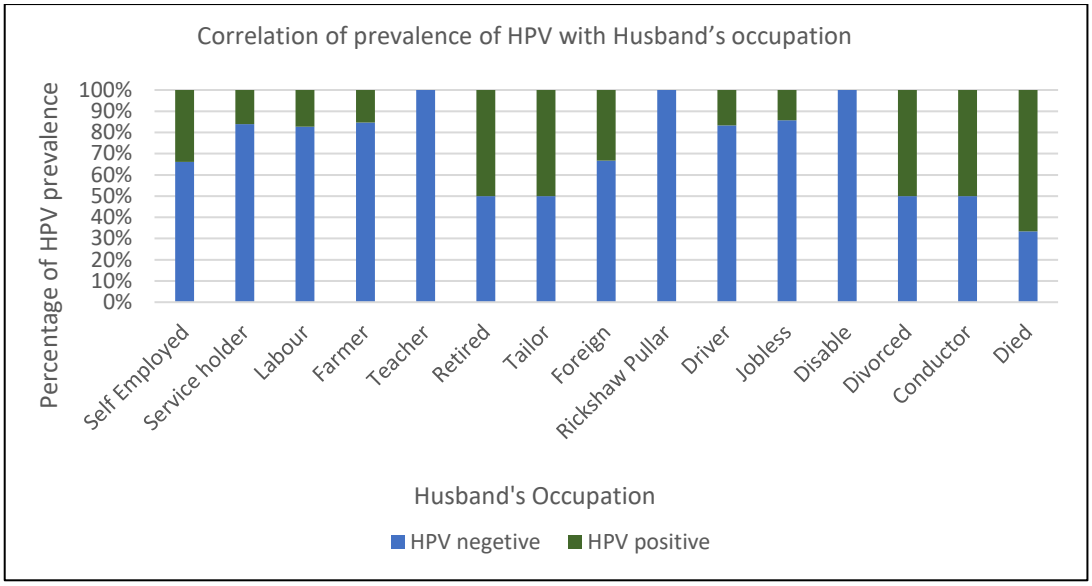

Figure 3.8: HPV prevalence with husband's occupation (p value = 0.00195).

From the analyzed data, we can clearly observe that around 52% wives of the self-employed husbands' are found to be HPV positive as well as the wives of husbands' earning a low to middle income show similar percentages. Whereas, a significantly lower prevalence was detected amongst the wives of moderately educated husbands'.

3.3.6 Correlation between prevalence of HPV with different types of symptoms of the women

In case of correlation between prevalence of HPV with different types of symptoms and any kind of complications of the women was also evaluated. It was thought that these symptoms might help for early detection of HPV infection so that treatment can start at earliest as possible.

Table 3.8: Prevalence of HPV with different types of symptoms of the women

Pain in lower abdomen	malaise	anorexia	Bleeding between period	cyst	Pain during intercourse	HPV Positive patients
No	No	No	No	No	No	17
Yes	No	No	No	No	No	17
Yes	No	Yes	No	No	No	1
No	Yes	Yes	No	No	No	1
Yes	Yes	Yes	No	No	No	1

3.3.7 Correlation between prevalence of HPV with age of women during their first intercourse

When it comes to find out the prevalence of HPV in women it becomes so necessary to check the starting time of their sexual life. That's why the correlation of prevalence of HPV with women's age of first intercourse were studied. As a matter of fact, it has been found that women who have started sexual life at an early stage from 12 to 25 has a chance to get infected with HPV and apparently these results were also statistically found significant (p value<0.05, at 95% CI).

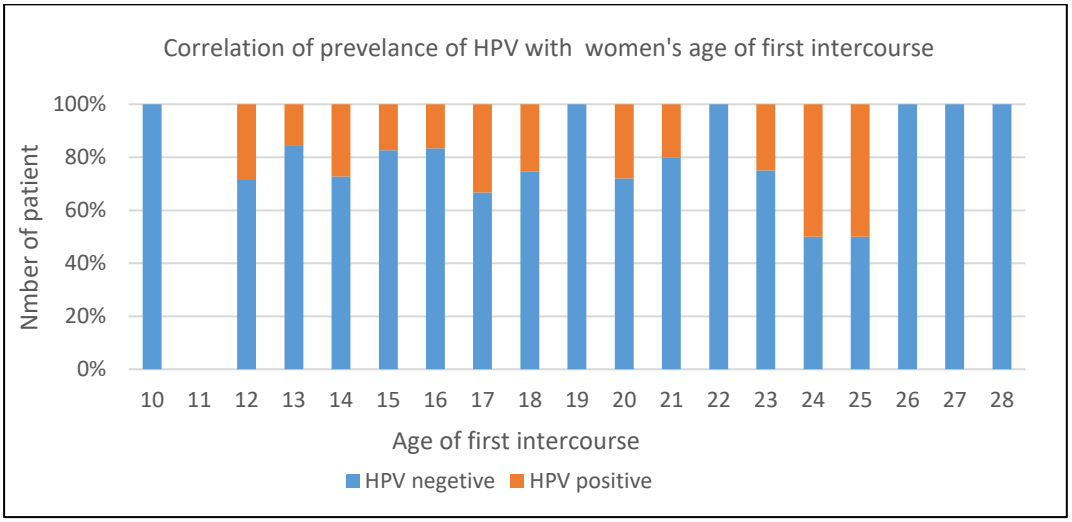

Figure 3.9: HPV prevalence with women's age of first intercourse (p value = 0.00914).

Prevalence of high-risk HPV also evaluated with the complications of the patients. The patients those had complications such as presence of genital warts, lower abdominal pain, backache, weight loss and pre-vaginal discharge with blood stained or foul smell and other cervical abnormalities, were found to be infected by high-risk HPV. HPV infection and its progression to cervical cancer is also related to initiation of sexual life at early ages and long term use of OCP. These were evaluated in cancer patients. All of the women who have developed cancer started their sexual life before the age of 18 years. Besides 42% of these women were using OCP for more than 3 years.

These findings showed that prevalence of high-risk HPV presumptively correlated with different predisposing factors like age, socio-economic status and behavior, use of long OCP for long time, complications etc.

3.4 To determine the association of *Chlamydia trachomatis* with HPV

Chlamydia trachomatis, an obligate intracellular sexually transmitted pathogen, is suggested to be a cofactor of cervical cancer. *C. trachomatis* often causes cervicitis. UsingCC3 (forward) and CC4 (reverse) primer, a conserved region of C. trachomatis plasmid pQH111L was amplified by standardized PCR system. Since the plasmid is highly conserved throughout different serovars, presence of this, therefore, confirms the existence of *C. trachomatis* in samples. As a matter of fact, the samples which were found positive for plasmid pQh111L, considered as infected with *C. trachomatis.* The amplified region of the plasmid was 201bp in size. After PCR optimization, gel electrogram pictures showed clear bands of this size which are shown in figure 3.10.

Such inflammation may predispose women to other STDs including genital HPV infection. *C. trachomatis* infection has been associated with the persistence of oncogenic infection.

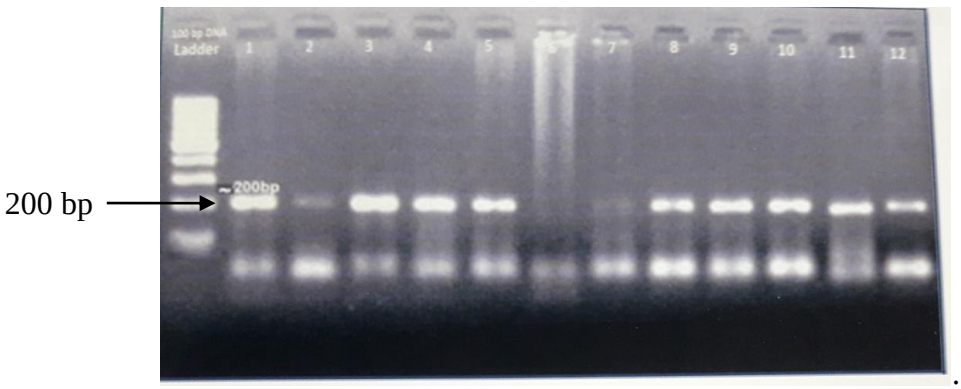

Figure 3.10: Agarose gel electrophoresis of *C. trachomatis* plasmid DNA-specific amplicon (201 bp) using CC3/CC4 primer pairs.

The aim of the study was to detect the presence of HPV (of both types) and *C. trachomatis* DNA in the samples. In this context, HPV status and the typing data for HPV-16 and HPV-18 were documented afterwards the number of positive samples for *C. trachomatis* was recorded for each group.

Here, it has been found out that approximately 41.73% cancer patients were infected with both HPV and *Chlamydia*; whereas 6.66% healthy women were found to be HPV and *Chlamydia* positive. All the data are summarized below in the table 3.9.

Table 3.9: Results of *C. trachomaits* DNA detection in the 112 cancer tissue samples and 170 cervical swab samples from apparently healthy women

Parameters	**HPV (+ve) samples from tissue**	**HPV(-ve) samples from tissue**	**Total number of tissue samples**	**HPV (+ve) samples from swab**	**HPV (-ve) samples from swab**	**Total number of samples**
No of samples	113	2	115	45	125	170
Samples which are tested positive for *C. trachomatis* DNA	48 (42.4%)	0 (0%)	48 (41.73%)	3 (6.66%)	4 (3.2%)	7 (4.12%)

3.4.1 Association studies between cervical cancer and *C. trachomatis*

The main objective of this study was to examine the correlation between cervical cancer and *C. trachomatis*. We hypothesized that there is a significant statistical relationship between these two factors. For this purposes, cervical cancer patients were considered as the study group and HPV negative, healthy, asymptomatic patients were considered as the control group. An odds ratio analysis was performed to determine the association according to the method described in Altman 1991. In terms of calculating the odds ratio, cervical cancer was considered as "exposure" while *C. trachomatis* infection was taken as the "outcome" as indicated table 3.10.

Table 3.10: Data for odds ratio analysis for association between cervical cancer and *C. trachomatis*

	Outcome (*C. trachomatis* infection)			Odds Ratio	22.6875
Exposure (Cervical cancer)	Result	Positive	Negative	95% CI	7.8284 to 65.7504
	Positive	48	64	Z statistic	5.750
	Negative	4	121	Significance level	$P < 0.0001$

As can be seen from the above table 3.10, the odds of *C. trachomatis* infection being present in cervical cancer patients are 22.6875. With a *p* value less than 0.0001, this result is found to be highly significant. In brief, our analysis finds strong correlation between *C. trachomatis* infection and cervical cancer.

3.4.2 Association studies between HPV DNA (possible cofounder) and *C. trachomatis*

At this stage of the analysis, to investigate the effect of a cofounding variable. As *C. trachomatis* and HPV can be co-transmitted, it is to consider that whether HPV DNA is variable that influences both *C. trachomatis* and cervical cancer progression. If so, then the apparent association between *C. trachomatis* and cervical cancer could be attributed to the co-transmission of *C. trachomatis* and HPV. To rule this out, another odds ration analysis was conducted to see if any statistical association exists between HPV and *C. trachomatis* infection, with HPV infection as exposure and *C. trachomatis* as outcome.

Table 3.11: Data for odds ratio analysis for association between HPV DNA and *C. Trachomatis* infection

	Outcome (*C. trachomatis* infection)			**Odds Ratio**	2.1607
Exposure	Results	Positive	Negative	95% CI	0.4643 to 10.0545
(HPV DNA)	Positive	3	42	Z statistic	0.982
	Negative	4	121	Significance level	*P* = 0.3260

As seen from the above table 3.11, with a p value of 0.3260 which means no significance association can be confirmed between HPV DNA and *C. trachomatis* infection. In other words, HPV infection does not have any confounding effect on earlier analysis on statistical relationship between cervical cancer and *C. trachomatis.*

3.5 Detection of serum proteins as biomarker by Western blot

As mentioned earlier the early diagnosis of a cervical cancer can improve the survival rate of the patient, by evaluating the detection of different serum proteins like HMGB1 (high mobility group box chromosomal protein 1), SCCA (Squamous cell

carcinoma antigen)/Serpin B3, CEA (Carcinoembryonic antigen), and Cytokeratin fragment (CYFRA) 21-1 could improve the diagnostic sensitivity and specificity.

3.5.1 Interpretation of results from the blotting membrane

As four different types of protein were considered as an antigen against them here four different types of multifunctional redox sensitive proteins were used as an antibody. After the SDS PAGE and western blotting following bands of proteins were found respectively. The pictures of the membranes are shown below accordingly in figure-3.11 to figure 3.14. In case of figure-3.11, blotting was done for the serum of patients (27-35) using Serpin B3, CEA and HMGB1 antibody, here 5 samples were found positive to have CEA protein of 77kDa M.W. and 8 proteins found positive to have Serpin protein 49kDa M.W.

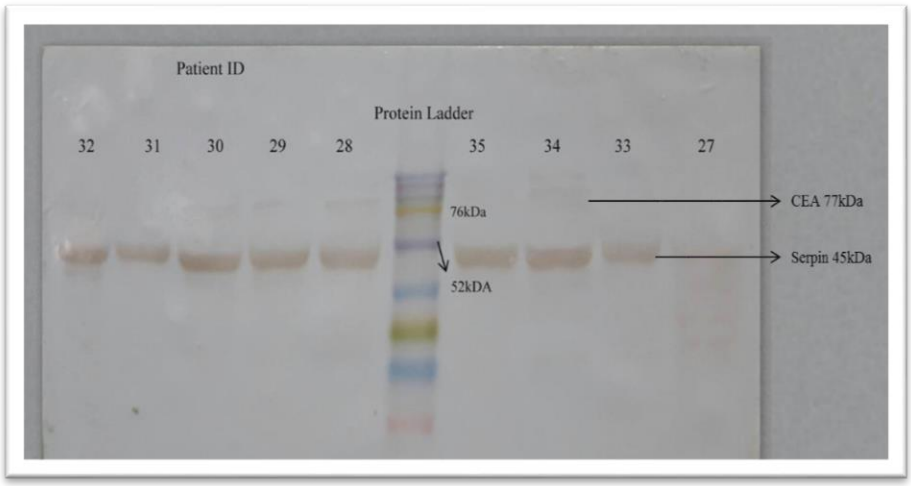

Figure 3.11: Western blotting of serum of patients (27-35) using Serpin B3, CEA and HMGB 1 antibody, here samples were found to have only CEA and Serpin B3 protein.

In case of below figure 3.12 blotting was done for the serum of patients (45-54) using Serpin B3, HMGB, and CEA antibody. Where 8 samples found positive having CEA protein of 77kDa M.W. only.

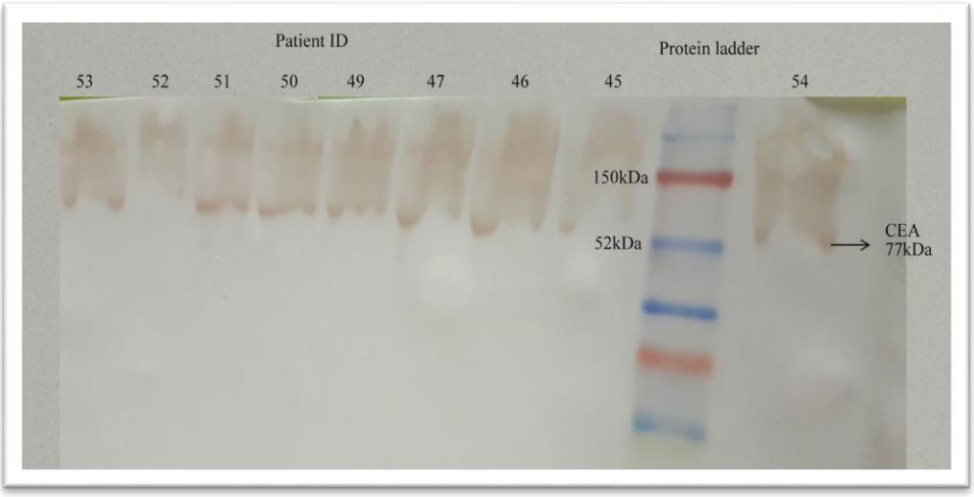

Figure 3.12: Western blotting of serum of patients (45-54) using Serpin B3, HMGB1, and CEA antibody. Where samples found positive having only CEA protein.

In case of below figure-3.13, blotting was done for the serum of patients (18-26) using Cytokeratin, HMGB1 and Transferrin antibody. Transferrin (77kDa) was used as an internal control. Here 9 samples found positive having Cytokeratin protein and 5 samples found positive to have HMGB1 protein.

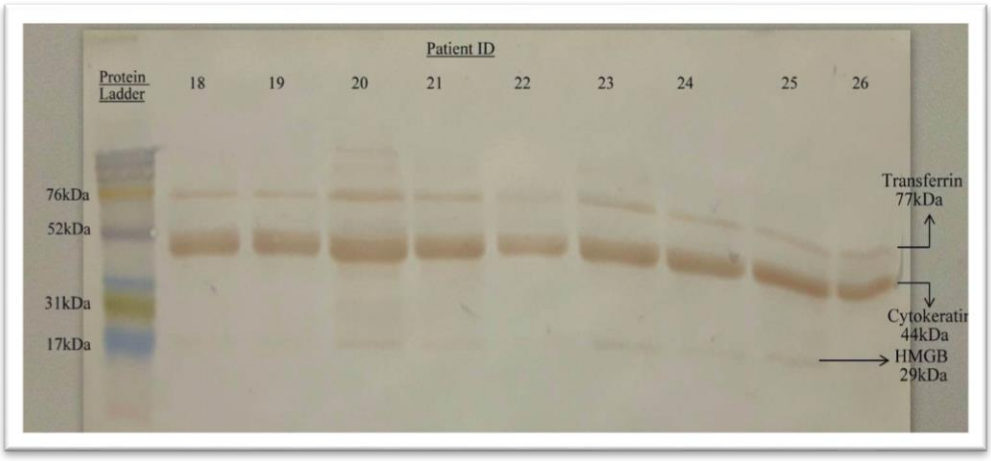

Figure 3.13: Western blotting of serum of patients (18-26) using Cytokeratin, HMGB1 and Transferrin antibody. Transferrin was used as an internal control. Here samples found positive having Cytokeratin and HMGB1 protein.

In case of below figure-3.14, blotting was done for the serum of patients (18-26) using Cytokeratin, HMGB1 and Transferrin antibody. Transferrin was used as an internal control. Here 9 samples found positive to have Serpin B3 protein, 8 samples found positive to have CEA protein and 4 samples found positive to have HMGB1 protein.

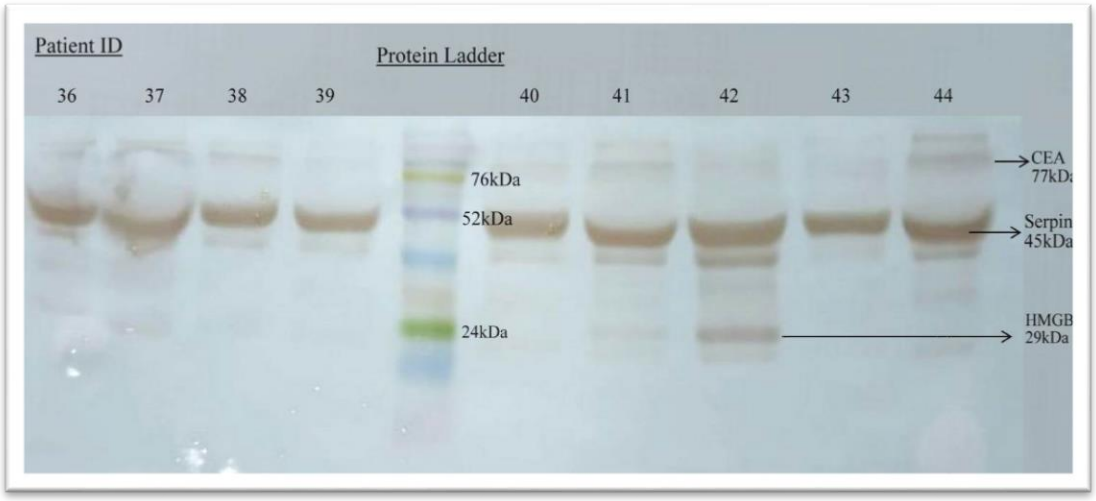

Figure 3.14: Western blotting of serum of patients (36-44) using CEA, HMGB1, and Serpin B3 antibody. Here samples found positive having CEA, Serpin B3, and HMGB protein.

3.5.2 Western blotting results of the cancer biomarkers

With the different parallel combinations of proteins, it would play a major role in the detection of cancer. As from Table 3.12 representing the positive responses of different types of proteins against different HPV types, statistically which is also found significant (p value < 0.0001). Here it reflects that among all the different combinations of proteins, most of them showed positive responses against HPV type 16, then some showed positive response for both HPV type 16 and18, and few showed responses against HPV type 18 only.

Overall, it can also be seen that among all four proteins CEA was the most prominent protein (49%) which showed the positive responses against cancer patient's serum. And then followed by SCCA (24%), HMGB1 (17%) and Cytokeratin fragment (10%).

Table 3.12: Positive responses with HPV types against protein combinations

No of different types of protein combination	HPV Type 16	HPV Type 18	HPV Type 16 and 18
HMGB (+), CEA (+) , SCCA (+), Cytokeratine (-)	6	1	1
CEA (+), Cytokeratine (+), SCCA (+), HMGB (-)	1	0	4
HMGB (-), Cytokeratine (-) and SCCA (+), CEA (+)	2	0	3
HMGB (-), CEA (-) and Cytokeratin (+), SCCA (+)	2	0	0
HMGB (+), CEA (+) and Cytokeratin (-), SCCA (-)	3	1	4
CEA (+), HMGB (-), Cytokeratine (-), SCCA (-)	6	4	6
HMGB (-), Cytokeratine (-), SCCA (-), CEA (-)	0	0	1

3.5.3 Statistical analysis of the immunoblot results

Below the graph figure 3.15 is also showing the difference of positive responses of different proteins against HPV type 16, 18 and both 16+18. After the chi square test, it was found significant having p value lower than 0.05.

After the chi square test for the detection of no. of proteins from the serum samples of cervical cancer patient it has found to be statistically significant having a p value lower than 0.01. Individually when considered the value of positive responses from serum containing HPV 16, 18, and both 16+18, it can be seen that among all the four proteins CEA was the predominant protein. Gradually positive responses were found for SCCA or Serpin B3 protein, then HMGB1 protein and least positive for Cytokeratin fragment protein.

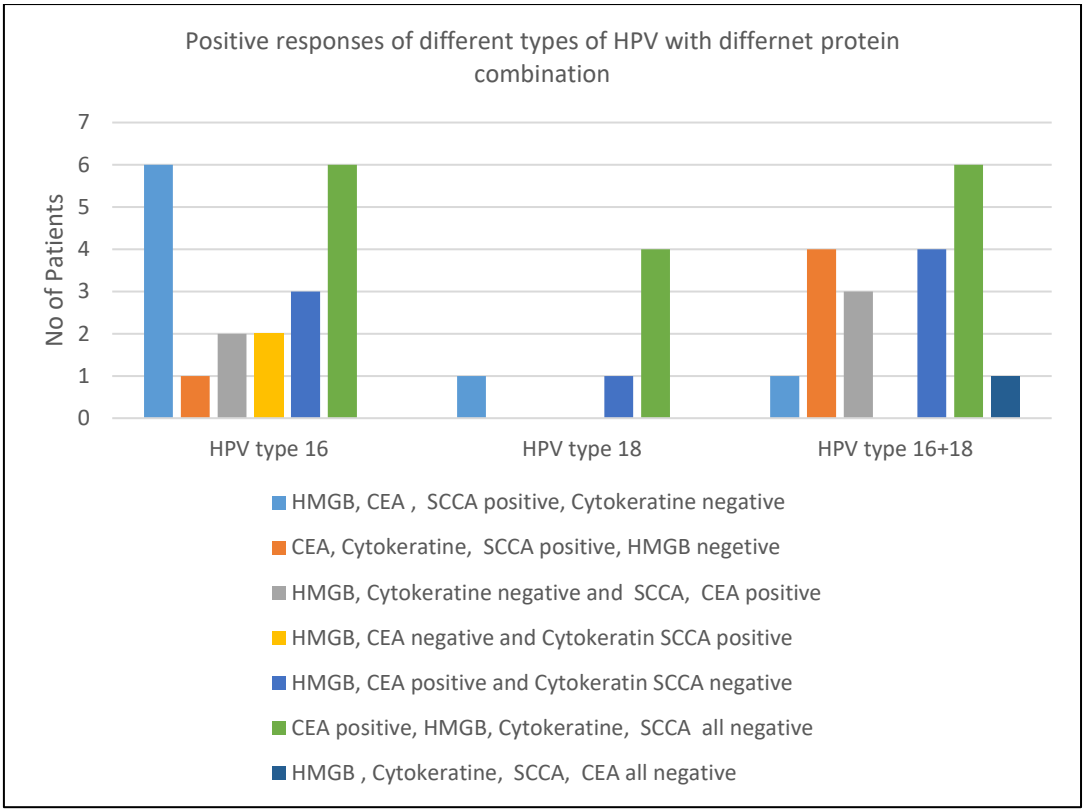

Figure 3.15: Positive response of protein combination with different types of HPV ($p < 0.05$).

In case of percentage of positive responses of proteins for HPV type 16, 18 and both 16+18 was also evaluated statistically which was also found to significance after the chi square test as p value was lower 0.001.

3.6 Antibody detection of HPV 6, 11, 16 and 18 from the serum of cervical cancer patients by ELISA

It has been difficult to study the humoral immune response to HPV, because these viruses are difficult to propagate in the laboratory. In that case it is important to better elucidate the natural history of HPV infection from the serological survey. The aim of the study was to find out the clinical responses in terms of antibody production by ELISA which might help early detection of cervical cancer.

Figure 3.16: ELISA plate containing the samples of serum of cervical cancer patients

For serological studies, papillomavirus L1 capsid proteins which are self-assemble into virus-like particles (VLPs) are effective antigens and can be used to detect type-specific humoral immune responses in patient sera [95, 156]. It is important to note that the recognized antibodies directed against VLPs are thought to neutralize infectious viruses in model systems [157]. However, neutralizing antibodies do not induce regression of established infection in animal models. So far, studies on serum antibodies to HPV capsids have focused on HPV seroprevalence in immunocompetent women [95, 156, 158, 159]. The aim of our study was to evaluate humoral (IgG) responses to intact HPV L1 VLPs of HPV- 6, -11, -16, and in women infected with HIV.

3.6.1 Interpretation of results of ELISA

Experimental samples value may be expressed relative to the values of control or non-immune samples, by calculation of a positive index as per the manufacturer's instruction.

After getting the OD value of the sera samples from the ELISA reader the results were interpreted, as a matter of fact considering the cut off value 17 patient's serum out of 77 were found to be positive meaning that they developed positive antibody activity. Statistically it was also found significant (p value <0.0001, at 95% CI).

Following are the results –

Table 3.13: Data for positive antibody activity by comparing the OD value along with positive index calculation

Total serum samples of cervical cancer patient	Calculated antibody activity	
	Positive (calculated value above 1)	Negative (calculated value below 1)
77	17	60
Significance (*p* value)	below 0.0001	
95% Confidence interval*	0.6463 to 0.8239	

* Confidence interval for every tested samples are shown as a range

Meanwhile, we have also set aside an intermediate condition for some values who are near to value 1 considering that along with the disease progression these patient's antibody activities might be increased afterward.

3.7 Molecular detection of specific gene fragments in cervical cancer samples by Polymerase Chain Reaction (PCR)

Three specific genes were chosen to be amplified for this study. *EGFR* gene fragments were amplified using one set of primers for each gene. Since *PIK3CA* and *KRAS* genes were indicated to harbor more mutation hotspot than *EGFR*, two sets of primers were used to amplify two different regions of the genes. In total, forty-six extracted DNA samples were used in this study.

Using the PIK3CA_1 primer set, the particular gene fragment was amplified for the 46 samples. PCR products were estimated to be sized approximately 700 bp on agarose gel electrophoresis (Figure 3.17-A). Using the PIK3CA_2 primer set, 46 samples were amplified. PCR products showed band on agarose gel electrophoresis at approximately 600bp (Figure 3.17-B).

Using the KRAS_1 primer set, extracted DNA samples were amplified. PCR products showed band on agarose gel electrophoresis at approximately 550bp (Figure 3.17-C). Using the KRAS_2 primer set, 46 samples were amplified. PCR products showed band on Agarose gel electrophoresis at approximately 550bp (Figure 3.17-D).

Using the *EGFR* primer set, DNA samples from 46 patients were amplified. PCR products showed band on agarose gel electrophoresis at approximately 600 bp (Figure 3.17-E).

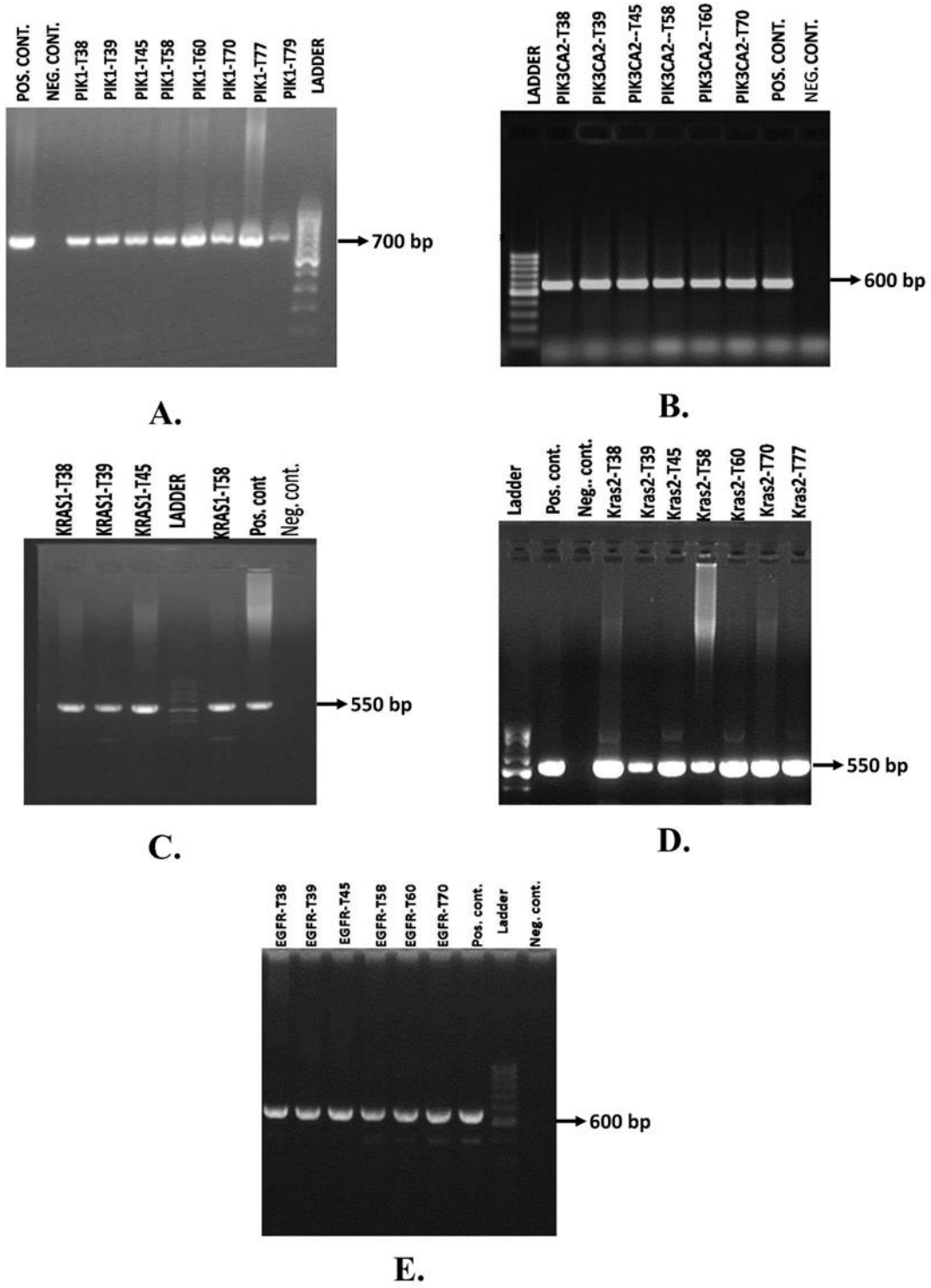

Figure 3.17: Agarose gel electrophoresis of PCR specific amplicon using A. PIK3CA_1, B. PIK3CA_2, C. KRAS_1, D. KRAS_2, E. EGFR primer pairs. 50bp ladder (Bioneer, USA) was used for comparison.

3.8 Sequence analysis for gene mutation study

After sequencing was carried out using purified PCR products and chromatogram quality checked and manual editing, clean sequence data analysis was done in three steps. They are:

- Matching with database
- Detection of change in base
- Checking with chromatogram to ensure the validity

All the samples were sequenced using both forward and reverse primers and then compared with each other to ensure correct sequencing.

3.8.1 Sequence data analysis by matching with database

In total, 230 gene fragments were sequenced (both strands). After obtaining sequence data, they were compared to the Gen Bank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST). All the sequences were matched with *Homo sapiens* chromosome primary assembly. *EGFR* gene is located on chromosome 7, *KRAS* gene is located on chromosome 12 and *PIK3CA* gene is located on chromosome 3.

3.8.2 Detection of mismatched base

All the BLAST results were carefully scrutinized to detect any mismatched base. Following is an example of a base mismatch found through BLAST analysis. There were two BLAST results, one showing no mutation and the other (Figure 3.18) having a mutation.

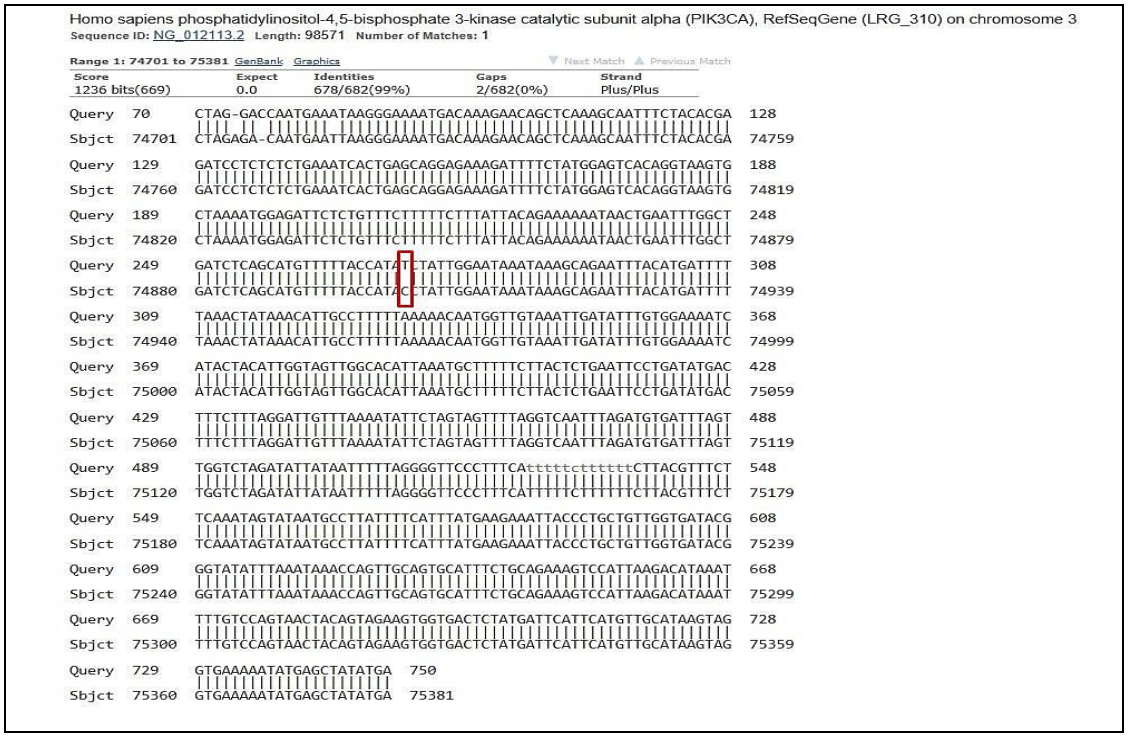

Figure 3.18: A BLAST analysis showing presence of a mutation. Red box indicates the mismatch.

3.8.3 Validation of mutation from chromatogram

The corresponding chromatogram was carefully inspected to ensure that the base change is not due to a sequence slippage but a valid mutation. If a clear peak was detected denoting one specific base, the sequence was acknowledged to be correct and base change was counted. Base changes for which clear single peaks did not exist were not counted as mutation. Mutation is confirmed from the chromatogram of second strand that was also sequenced.

3.8.4 Result of sequence analysis

Using BLAST search, in total 39 mutations were found in 28 patients among the 46 patient samples (Table 3.14). Rest of the samples did not harbor any mutation along the region were sequenced.

Table 3.14: Detailed result of sequence analysis

Patient ID	Gene	Mutation	Mutation Type	Position (Chromosome) GRCh38.p7	Intron/ Exon	Comment
T70	*PIK3CA*	T >A	Transversion	Chr3 179218237	Exon	523 Missense L>I
T108	*PIK3CA*	T >A	Transversion (htg)	Chr3 179234443	Exon	3'UTR
T70	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T104	*PIK3CA*	T > G	Transversion	Chr3 179218439	Intron	
T108	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T38	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T113	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T44	*PIK3CA*	G> A	Transition	Chr3 179218352	Intron	
T52	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T90	*PIK3CA*	C >T	Transition	Chr3 179218425	Intron	
T53	*PIK3CA*	T > G	Transversion	Chr3 179218439	Intron	
T100	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T40	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T47	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T51	*PIK3CA*	T>A	Transversion	Chr3 179234011	Intron	
T71	*PIK3CA*	T>A	Transversion	Chr31792340 11	Intron	

Patient ID	Gene	Mutation	Mutation Type	Position (Chromosome) GRCh38.p7	Intron/ Exon	Comment
T101	*PIK3CA*	T>A	Transversion	Chr3179234011	Intron	
T90	*PIK3CA*	C >T	Transition	Chr3179234223	Exon	Synonymous I>I
T55	*PIK3CA*	T>A	Transversion	Chr3179234446	Exon	3'UTR
T90	*PIK3CA*	T>A	Transversion	Chr3179234447	Exon	3'UTR
T108	*PIK3CA*	C >T	Transition	Chr3179234232	Exon	1025 T>T Synonymous
T38	*PIK3CA*	C >T	Transition	Chr3179234232	Exon	1025 T>T Synonymous
T70	*PIK3CA*	C >T	Transition	Chr3179234232	Exon	1025 T>T Synonymous
T97	*PIK3CA*	C >T	Transition	Chr3179234232	Exon	1025 T>T Synonymous
T45	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T58	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T60	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T97	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T106	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T109	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T115	*EGFR*	G> A	Transition	Chr7 55170575	Exon	3'UTR
T51	*EGFR*	G> A	Transition	Chr7	Intron	

Patient ID	Gene	Mutation	Mutation Type	Position (Chromosome) GRCh38.p7	Intron/ Exon	Comment
T51	*EGFR*	G> A	Transition	55170193 Chr7 55170210	Intron	
T47	*EGFR*	Insertion (A)	Addition	Chr7 55170230	Intron	
T47	*EGFR*	A>G	Transition	Chr7 55170332	Exon	636 Mis-sense M>V
T91	*KRAS*	G>A	Transition (htg)	Chr12252452 46	Intron	
T84	*KRAS*	A>C	Transversion (htg)	Chr12252271 121	Intron	
T108	*KRAS*	A>C	Transversion (htg)	Chr12252271 121	Intron	
T110	*KRAS*	A>C	Transversion (htg)	Chr12 252271121	Intron	

Note: Htg = heterozygous, chr = chromosome

In summary, our data show that among 46 samples, 18 did not harbor any mutation (39.13%), 22 samples harbored mutation in one gene fragment (47.83%) whereas 6 samples harbored mutations in more than one gene fragments (13.04%) (Figure 3.19).

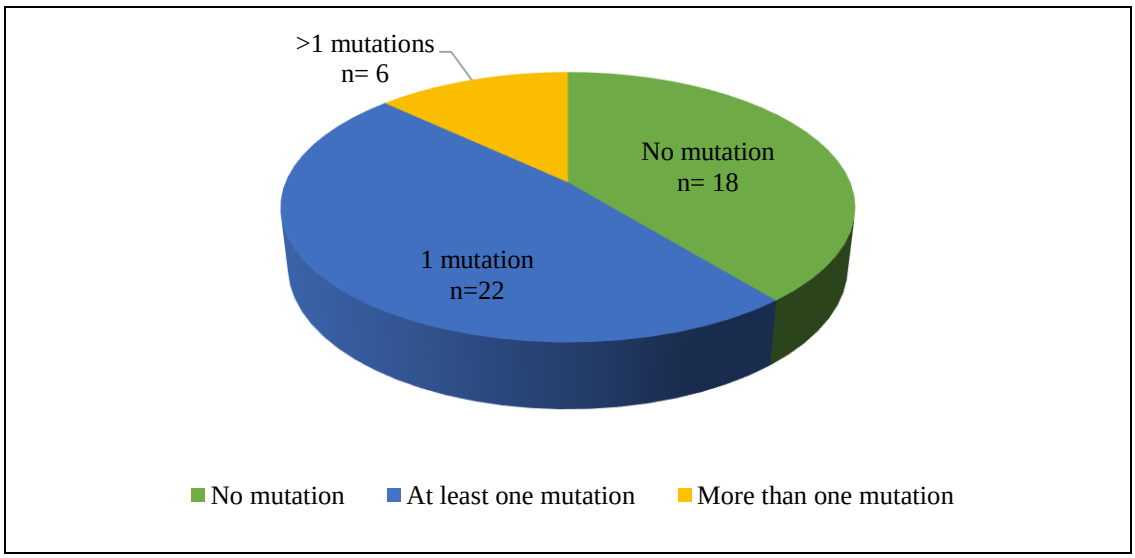

Figure 3.19: Distribution of cases by presence of mutation.

3.8.4.1 Individual gene mutation result

Amongst the 46 samples, it has been found that *PIK3CA* was the most frequently mutated gene (in 52.17% patients), *EGFR* gene was the second most mutated gene (in 23.91% patients) and finally *KRAS* gene was found to be the least mutated in the tested patients (in 8.7% patients) (Table 3.15).

Table 3.15: Individual gene mutation result

***KRAS* Gene**	***EGFR* Gene**	***PIK3CA* Gene**
4 in 46 samples	11 in 46 samples	24 in 46 samples
8.7%	**23.91%**	**52.17%**

3.9 Mutation analysis

3.9.1 Analysis of effect of exonic and intronic mutation on gene function

Among the 39 mutations, 17 were unique. These unique mutations were analyzed using Variant Effect Predictor tool [141]. All 17 different mutations in the 3 target genes showed significant low LoFtool scores (Table 3.16) which can be used to predict that all these mutations can have probable damaging effects and make the patients more vulnerable to carcinogenesis and metastatic development.

Table 3.16: Prediction of the putative effect of the mutation on gene function.

Gene	Mutation	Intron/ Exon	Position (Chromosome)	LoFtool Score	Mutation Effect
EGFR	G> A	Exon	Chr7 55170575	0.0455	Probably Damaging
	G> A	Intron	Chr7 55170193	0.0455	Probably Damaging
	G> A	Intron	Chr7 55170210	0.0455	Probably Damaging
	Insertion (A)	Intron	Chr7 55170230	0.0455	Probably Damaging
	A>G	Exon	Chr7 55170332	0.0455	Probably Damaging
KRAS	G>A	Intron	Chr12 25245246	0.19	Probably Damaging
	A>C	Intron	Chr12 252271121	0.19	Probably Damaging

Gene	Mutation	Intron/ Exon	Position (Chromosome)	LoFtool Score	Mutation Effect
PIK3CA	T >A	Exon	Chr3 179218237	0.268	Probably Damaging
	T >A	Exon	Chr3 179234443	0.268	Probably Damaging
	C >T	Intron	Chr3 179218425	0.268	Probably Damaging
	T > G	Intron	Chr3 179218439	0.268	Probably Damaging
	G> A	Intron	Chr3 179218352	0.268	Probably Damaging
	T>A	Intron	Chr3 179234011	0.268	Probably Damaging
	C >T	Exon	Chr3 179234223	0.268	Probably Damaging
	T>A	Exon	Chr3 179234446	0.268	Probably Damaging
	T>A	Exon	Chr3 179234447	0.268	Probably Damaging
	C >T	Exon	Chr3 179234232	0.268	Probably Damaging

3.9.2 Exonic mutation frequency

Seventeen mutations were found to be exonic among the 39 mutations and rest others were intronic. Among the exonic mutations, two mutations were found to be non-synonymous, one was found in the *PIK3CA* gene and other one in the *EGFR* gene. None of the mutations in the *KRAS* gene were exonic. *PIK3CA* gene was found to contain a high amount of exonic mutations (72.7%) than the *EGFR* gene (37.5%) (Table 3.17)

Table 3.17: Frequency of mutation found in exon

Gene	Total mutation	Exonic mutation	Number of non-synonymous mutation	Intronic mutation	% of exonic mutation
PIK3CA	24	9	1 out of 9	15	37.5%
EGFR	11	8	1 out of 8	3	72.7%

3.9.3 Determination of the location of mutant amino acid in the protein

Using Mutation Mapper tool [146] the particular domain where the target mutant amino acid is located were found. In EGFR protein, mutant amino acid (valine) is

present in a functional domain named Growth Factor Receptor 4 domain. Missense mutation of PIK3CA protein was located in the beginning of the PIK domain, which is conserved in all PI3 and PI4-kinases and role of this domain unclear but it has been suggested to be involved in substrate presentation [160].

3.9.4 Analysis of the effect of non-synonymous mutation on protein

SIFT scores for the non-synonymous mutations are found to be high and Poly Phen scores are found to be lower for both genes (Table 3.18), predicting the structure and function of the proteins can retain its natural states even though the mutation occur to the change the particular amino acid.

Table 3.18: Prediction of the effect of the non-synonymous mutation on protein structure and function.

Gene	Mutation	Intron/ Exon	Position (Chromosome)	SIFT Score	PolyPhen Score
EGFR	G> A	Exon	Chr7 55170575	0.5 (tolerated)	0 (benign)
PIK3CA	T >A	Exon	Chr3 179218237	0.26 (tolerated low confidence)	0.001 (benign)

Due to the low delta G value for the both of the non-synonymous mutations, MuProtool predicted that both of the protein structures may lose its stability upon the accumulation of the particular variant amino acid instead of the wild-type one (Table 3.19).

Table 3.19: Mutant protein's stability prediction.

Gene	Mutation	Intron/ Exon	Position (Chromosome)	MuPro Score (Delta delta G value)	MuPro Prediction
EGFR	G> A	Exon	Chr7 55170575	-1.2135263	Decrease stability of protein structure
PIK3CA	T >A	Exon	Chr3 179218237	-1.0116756	Decrease stability of protein structure

3.9.5 Detection of change of amino acid and its effect in protein 3D structure

All the nucleotide sequences were translated into amino acids to find out whether there any crucial amino acid was changed due to mutation, and also if such amino acid change may lead to significant change in 3D protein structure. Structure of mutated proteins was determined by homology modeling using the tool SWISS-MODEL [147].

Among the 2 non-synonymous mutations, first one was in *EGFR* gene (chromosome 3) at position 55,170,332. The A to G base transition has resulted in an amino acid change from methionine to valine at amino acid position 636. This amino acid change was used to predict change in protein structure using SWISS-MODEL and as a result, no significant structural difference has been observed due to that particular amino acid substitution.

The second mutation was in *PIK3CA* gene (chromosome 3) at position 179,218,237. Here, the T to A base transition has resulted in an amino acid change from leucine to isoleucine at amino acid position 523. This amino acid change caused a slight change in PI3 and PI4-kinases domain in 3D structure (Figure 3.20).

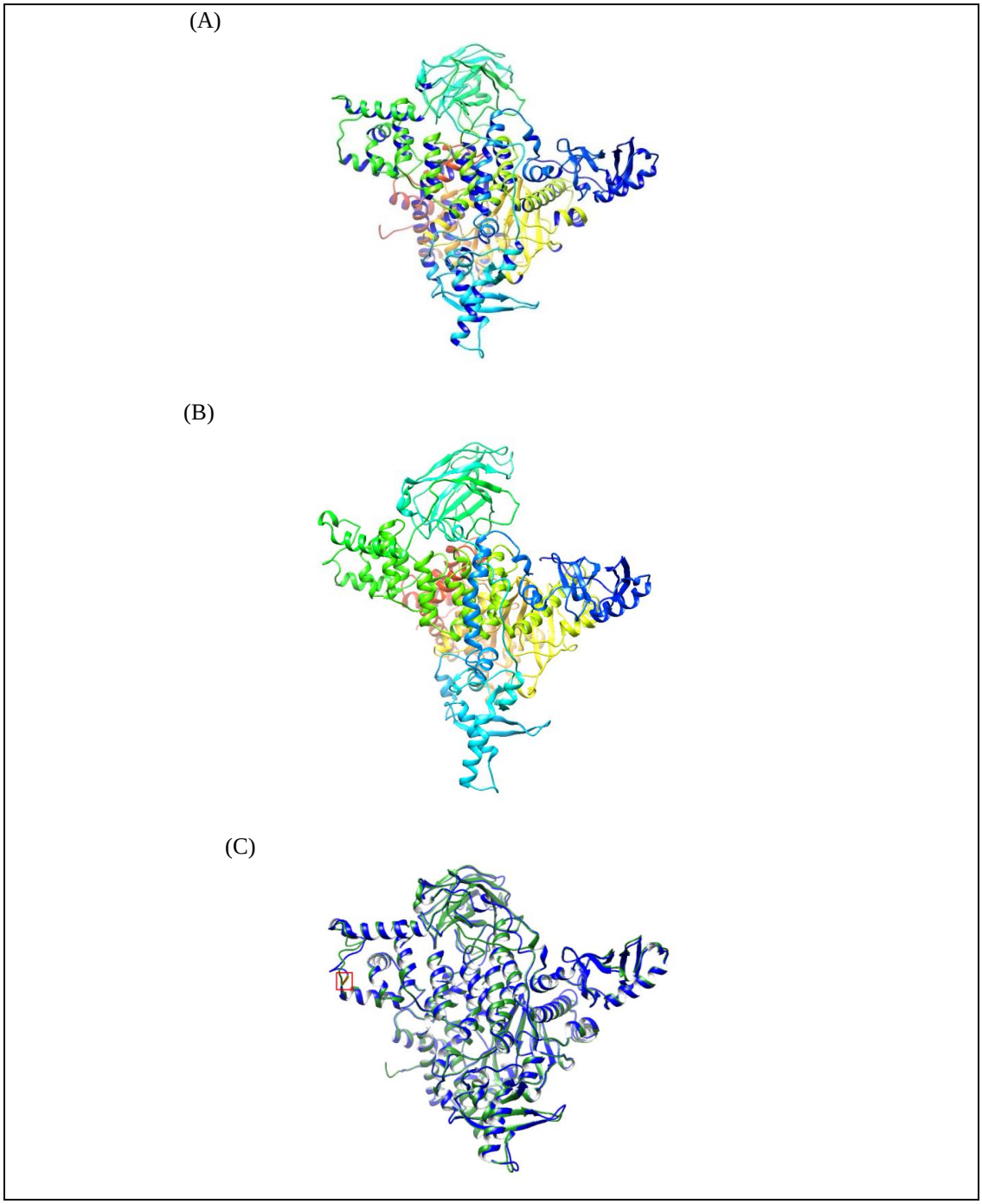

Figure 3.20: 523L>I mutation in *PIK3CA*. (A) The wild-type protein, (B) the mutated protein and (C) the wild-type and the mutated protein are overlapped where the marked red box shows the mutation position in PIK3CA protein structure.

CHAPTER FOUR

DISCUSSION AND CONCLUDING REMARKS

4. Discussion

Cervical cancer is one of the most frequent cancer in women in 2012 representing 7.9% of all female cancers [161]. Approximately 90% of the 270,000 deaths from cervical cancer in 2015 occurred in low and middle-income countries [161]. This is the most common cause of female death by cancer in 43 countries. The estimated global economic burden of cervical cancer in 2009 was 3 billion US dollars.

The impact of cervical cancer is more conspicuous in developing countries like Bangladesh. This is mainly because of the existence of a huge knowledge gap regarding the risk factors of this disease. Furthermore, lack of data makes it impossible to understand the actual scenario of cervical cancer in our country. Our study sought to bridge this knowledge gap, being the first one of its kind in the subcontinent.

According to Walboomers (1999), about 99.7% of invasive Cervical Cancer (CC) cases worldwide are associated with infection by HPV. Cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries. Vaccination against these HPV and hepatitis B viruses could prevent 1 million cancer cases each year [54]. HPV is recognized as the most important risk factor for cervical cancer for last 15 years [162]. World health organization recognized sexually transmitted HPV infection as a modifiable and avoidable risk factor.

4.1 Molecular detection and genotyping of HPV DNA

Infection with distinct types of human papillomavirus (HPV) is the major etiologic factor in cervical carcinogenesis. This causal relationship has been exploited for the development of molecular technologies for viral detection to overcome limitations linked to cytologic cervical screening [163]. HPV DNA testing was recently approved by the Food and Drug Administration for use as an adjunct to cytology for cervical cancer screening, where consensus was reached based on a literature review, expert opinion, and unpublished results from large ongoing screening studies [164]. HPV DNA can be detected in nearly all cervical carcinomas [165] and increasing HPV prevalence rates are found with increasing severity of cervical dysplasia [166]. Due to the strong correlation between HPV and CC, various efforts have been made to improve the sensitivity of the methods to detect HPV type in samples from women

with abnormal genital cytology. HPV detection and typing has become an essential tool for diagnostics and management of HPV-related disease [167-169]. It has been shown in this study that although there were no abnormalities in the cellular cytology but high-risk HPV can be present there.

Bangladesh has a population of 54.38 million women ages fifteen years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 11,956 women are diagnosed with cervical cancer and 6,582 die from the disease (HPV Information Center). Cervical cancer ranks as the second most frequent cancer among women between 15 and 44 years of age in Bangladesh [19]. In the Southern Asia, about 7.9% of women in the general population are estimated to harbor cervical HPV infection at a given time and 82.8% of invasive cervical cancers are attributed to HPV 16 or 18 [170]. Vaccination program in Bangladesh is in its primary steps, and vaccine is costly, with approximately 1,600 BDT per dose.

Only Pap test or VIA test should not be used solely as screening test for cervical cancer. HPV DNA test must be performed to ensure the status of the patients. A patient having both Pap or VIA test along with HPV DNA test can only be managed correctly.

Data on the association of HPV with CC or its prevalence in apparently healthy women in Bangladesh are rare or absent. No such research has been undertaken to find out the distribution subtype of HPV in the cancerous cases. In this study, we have tested the epidemiology of HPV in Bangladesh. Our results showed that the HPV prevalence is 29.51% in Bangladesh (121/410) in healthy women, and the prevalence of high-risk HPV was 19.53%. The distribution of HPV prevalence exhibits regional differences. Conducted a multi-center, population-based study between February 2015 to May, 2018 in different part of Bangladesh

When demographical data were analyzed it has been observed that correlation of HPV prevalence age of women, education level of the women and occupation of the husband of the women were found statistically significant whereas correlation of HPV prevalence with the age of women during first intercourse, economical status, use of contraceptives and women from different regions of Bangladesh and mild symptoms of women were found statistically non-significant. In previous studies it has also been seen that the presence of HPV DNA in the husbands conveyed a fivefold risk of

cervical cancer to their wives. Presence of antibodies to *C. trachomatis* and an early age at first sexual intercourse with husband were also associated with cervical neoplasia in the wife [171]. In Spain and Colombia where a study of cervical cancer was conducted to assess the relationship between cervical cancer and exposure to human papillomavirus (HPV), selected aspects of sexual and reproductive behavior, use of oral contraceptives, screening practices and smoking was also considered as a risk factor [172]. After adjustment for these variables and for the wife's pack-years of smoking, the husband's smoking was moderately associated with cervical cancer in his wife was also found in Spain. Men who report multiple sexual partners or who are carriers of HPV DNA may be of high-risk HPV types and may place their wives at a risk of developing cervical cancer [171].

In addition, the distribution of HPV subtypes also exhibited regional differences. Previous studies have indicated that HPV16, 18, 31, 33, and 58 are the most common types in Europe; the prevalence is 2.3%, 0.7%, 0.6%, 0.4%, and 0.4%, respectively. In northern America, HPV16, 53, 52, 18, and 39 are the most common types; the prevalence is 3.5%, 1.1%, 1.0%, 1.0%, and 0.9%, respectively. In Africa, HPV16, 52, 18, 58, and 31 are the most common types; the prevalence is 2.7%, 1.8%, 1.6%, 1.6%, and 1.3%, respectively, and in Asia, HPV16, 52, 58, 18, and 56 are the most common types, with the prevalence is 2.6%, 1.2%, 1.0%, 0.8%, and 0.8%, respectively [26]. In Bangladesh HPV16, 18, 31, 33, 70, and 69 are the most common types, with the prevalence of high-risk HPV 16 and 18 is identified in 28.5% , and intermediate risk HPV 31, 33, 70 and 69 in 1.21% [173]. HPV-16 and -18 subtypes are the most commonly seen subtypes in the world. HPV-33, -45, -52, and -58 subtypes are more prevalent in Asia than anywhere else in the world, especially in China [174]. Our results showed that the highest frequency of the low-risk HPV subtypes was for HPV-6, -70, -31, -33 and -69 and the prevalent high-risk subtypes were HPV-16, -18, and -16+18 combination. High-risk HPV subtypes confer a high risk of cervical cancer [175]. Based on our findings, HPV screening systems should focus on HPV-33, -31, -69 and -70 equally to HPV-16 and -18 in Bangladesh. In the present study, infection with multiple subtypes accounted for 18.18% of the HPV infections and 5.36% of all samples. The distribution of HPV subtypes of infected individuals in the different age ranges are already reported and the high-risk HPV infections (include low-high-risk

and high-high-risk) accounted for 37.19% of the HPV infections and 10.9% of all samples, [176].

Moreover, the distribution of HPV subtypes and their prevalence exhibited age related differences. Two of the most common age ranges for HPV infection were those under 14 and over 60 years old, which was similar to that observed in previous studies in China [173]. The main reason may be that the immune system against HPV in young women is immature and the physiological and immunological disorders associated with hormone fluctuations during the menopausal transition of older women.

There are several limitations to the data in this study. First, the total samples considered from the age group of 20 and that over 65 were much lower than the total samples considered from other age groups, which might lead to prevalence data from the age group of 20 and that over 65 being unreliable. In addition, the regional prevalence was calculated based on all samples received from this region, and not based on the entire population of this region. Similarly, the age-specific prevalence was calculated based on all samples received from these age groups, and not based on the entire population in these age groups. We are not aware of the number of remaining women that had HPV in our region with- out being diagnosed, and this could introduce a certain bias and lead to increase in the prevalence. Our results showed that HPV type 16 and type 18 prevalence is 29.5% in Bangladesh (121/410), which is obviously higher than the 8.3% prevalence in Asia [26].

In conclusion, this study shows that this prevalence of HPV type 16 and type 18 varies significantly among different ages and regions in Bangladesh. These results can serve as a valuable reference to guide cervical cancer screening and HPV vaccination programs in Bangladesh. Additionally, HPV testing in cervical cancer screening programs has clinical significance for women under 20 and over 60 years of age, and development of a vaccine targeting HPV-16, -18, -33, -52, and -58 subtypes is important.

In case of cervical cancer tissue samples, 113 samples out of 115 cervical cancer tissue samples were found to be positive for HPV DNA. In this study, 2 samples which were taken from cancer patients showed negative result in nested PCR. The reason behind it might be the L1 region, which was targeted for amplification might not present or other genes of HPV except L1 were integrated within the host genome

during infection. That's why GP5/GP6 primer were failed to detect HPV. These samples require further analysis with other primers that would amplify other genes e.g. E6/E7.

The genotype of HPV was detected to know whether the virus is of High- or Low-Risk group. Total 115 HPV positive samples for cervical tissue were analyzed by multiplex type specific PCR. Among them 44 samples were positive for both HPV-16 and HPV-18. Besides these, 54 samples contained only HPV-16 and 14 samples contained only HPV type-18. There were only 1 sample for each that were typed as HPV -70 and HPV -31.

From this study it has been found that, high risk HPV can be found in VIA negative women. But when the endo-cervical swab samples were analyzed by PCR for the presence of HPV, it was found that 11 of the females were infected with HPV. HPV-18 is the most prevalent among asymptomatic individuals. On the other hand, it has found that in cancer patients HPV-16 and both HPV-16 and 18 are present predominantly.

4.2 Correlation between the presence of high-risk HPVs and several predisposing factors

Correlation between the presence of high-risk HPVs and several predisposing factors were also analyzed in this study. It has been figured out from this study that HPV remains in a pre-cancerous stage at 15-30 years age range and takes decades to progress into cancer. Women who have started their sexual life before the age of 18 years are prone to HPV infection and they have developed cancer after 40 years of age. If these women who are in pre-cancerous stage can be treated properly their lives could be saved.

Socio-economic status of patients was also evaluated. It has been deduced from this study that the illiterate females of lower socio-economic status are more prone to be infected by HPV. This can be because of inability to afford the cost of cervical cancer screening test and vaccination and being unhygienic. While socio-economic status increases, chance of infection decreases. Prevalence of high-risk HPV also evaluated with the complications of the patients. The patients those had complications such as presence of genital warts, lower abdominal pain, backache, weight loss, pre-vaginal

discharge with blood stained and foul smell and other cervical abnormalities were found to be infected by high-risk HPV dominantly.

4.3 Statistical association between *C. trachomatis* and cervical cancer

C. trachomatis has been considered as plausibly being a cofactor for the development of cervical cancer [177]. Despite that plausibility, there is still so scientific consensus about whether there is a significant association between cervical cancer and *C. trachomatis.* The standard detection method of C. trachomatis has been the cell culture [178]. However, several clinical and technical factors can lead to false negative results in the culture system [179]. In addition, this method is time consuming, expensive and inapplicable for screening a large number of samples at a time.

Most of the previous studies conducted to investigate the correlation between *C. trachomatis* and cervical cancer, were based on seroepidemiology. Antigen assay, which is commonly used for *C. trachomatis* detection, appears to be an easier method but it is considerable less sensitive than cell culture. Several reports [180] indicated that PCR could serve as a good alternative to both tissue culture as well as antigen assay. On that premise, we detected *C. trachomatis* using a standardized PCR based detection method. Using a set primer of primers, a region of approximately 201 bp on the plasmid was amplified for subsequent detection. The plasmid is usually present in multiple copies in the cell of *C. trachomatis*. Differences in *C. trachomatis* prevalence might also reflect different patterns of sexual behavior or the lower frequency of barrier contraceptive method used in different geographical setting [181].

A correlation between *C. trachomatis* infection and dysplastic or neoplastic cervical abnormalities has been observed by several authors [182, 183] and this finding has led to the hypothesis that *C. trachomatis* is a cofactor in HPV related carcinogenesis. As indicated above, we wanted to investigate whether there is any statistical relationship between cervical cancer and *C. trachomatis* infection given the lack of scientific consensus on the matter. For this purpose, we took cervical cancer patients as our study group, and to confirm the presence of HPV in the tissue samples,

We took HPV negative, healthy, asymptomatic individuals as our control group. Individuals comprising the control group did not have any sign of cancer development or any sorts of sexually transmitted infection. Individuals comprising both study and

control groups were sexually active women of similar age group. Data on their HPV status was extracted from previous gray literature publications from the laboratory [184].

As mentioned earlier, we detected the presence of *C. trachomatis* using a DNA based detection method in both study and control groups. The cervical cancer and *C. trachomatis* infection data was then used to run an odds ratio (OR) analysis. At 95% confidence interval (CI), the range for this was found to be from 7.83 to 65.75, which is a measure of the variance. The significance level or *p* value for this study was found to be almost equal to zero (p value<0.0001). Meaning, not only did our study demonstrate a strong degree of correlation between cervical cancer and *C. trachomatis,* the high degree of significance indicates that the results are on solid statistical footing.

4.4 Antibody detection of HPV 6, 11, 16 and 18 in the serum of cervical cancer patients by ELISA

As we know the difficulty to study the humoral immune response to HPV, it becomes necessary to elucidate the natural history of HPV infection from the serological survey and also to disclose antigen determinants which are useful for vaccine development and new approaches which are needed to correlate the antibody response to the disease outcome [185]. In this case, we have so far found that this antibody production in the sera against the HPV antigen might be considered as an effective diagnostic tool by ELISA for early detection [186]. Here a significant amount of patients' sera showed positive antibodies which is 17 out of 77 cancer patient's sera samples.

Due to the limited number of sera both for cervical cancer and control, tested in the present study, it is evident that this ELISA system needs validation in large-scale population studies, especially for the detection of HPV pan-reactive antibodies in people infected by genotypes different from HPV6, 11, 16, and 18. Nevertheless, in view of the known cross-reactivity between the most common HPV genotypes circulating in Europe [187], this ELISA should be useful in monitoring the pre-existing serological status of women who receive HPV vaccination with the recently introduced prophylactic VLP-based vaccines and also could help in designing and improve the treatment protocol and as well as establish a new vaccination program.

4.5 Detection of serum proteins as biomarker by Western Blot

In developed countries, organized screening programs have significantly reduced cervical cancer incidence, however cytology-based screening has several limitations. In that case, pap tests are required which is costly retesting or diagnostic work-up by colposcopy and biopsy. In low-resource countries, it has been difficult to establish and sustain cytology-based programs. That is why advancement in understanding human papillomavirus biology and the natural history of human papillomavirus-related pre-cancers and cancers have led to the discovery of a range of novel biomarkers in the past decade. In this study, we have used several proteins as a new biomarker for primary screening, and diagnosis [188].

Cervical cancer prevention is at a transition from cytology-based screening programs to HPV-based prevention. Primary prevention is done by using vaccines and secondary prevention is done by using a highly sensitive HPV DNA test which is extending screening intervals and becoming crucial for these programs to work [189]. As a result, new biomarkers will be important to decide who among the HPV-positive women needs to be referred for further evaluation or treatment. Large studies are currently underway for various triage biomarker candidates [190].

In this study we have only seen the presence of the HMGB1 (high mobility group box chromosomal protein 1), SCCA (Squamous cell carcinoma antigen), CEA (Carcinoembryonic antigen), and Cytokeratin fragment (CYFRA) 21-1 proteins in the cancer patient's serum. And it has been so far seen that combinations of different proteins measurement could confirm the cervical cancer.

This study showed that the highest percentage of patient who showed positivity against these protein was infected with HPV type 16.

It was the limitation of the study that we could not quantify the proteins at different levels. As an ideal tumor marker should have a high sensitivity and a high specificity in order to discriminate between patients with cancer and those with benign conditions or healthy controls, and should also provide information related to tumor burden and activity. The clinical relevance of a serum tumor marker should be validated in a large prospective study or a meta-analysis of small-scale retrospective/prospective studies before routine use. Till today there is no validated tumor marker is currently available yet for the diagnosis, prognostic evaluation, and

treatment monitoring and follow-up of patients with cervical cancer [191]. As a matter of fact, it can be expected that the first screening programs based on primary HPV testing and new biomarkers as secondary tests would be implemented in a few years. It will be important to reserve treatment for those women who are at risk of developing cancer, rather than treating any high-grade lesion. Prospective biomarkers may play an important role in these therapy decisions [188].

4.6 Detection of mutation and polymorphism in marker genes of cervical cancer

Mutation that can induce neoplastic transformation is known as oncogenic mutation and can produce an abnormal protein or change expression level of the protein coded by the gene harboring the mutation. This abnormal or overexpressed protein can be a potential target of drugs. These drugs can eventually destroy cancer cell or modify the disease process. In this perspective, researchers are trying to find out mutation specific for certain cancer in last few decades. A classic example is the translocation of gene segment between two chromosomes resulting in BCR-ABL fusion protein found in chronic myeloid leukemia (CML). A Tyrosine kinase inhibitor named imatinib which inhibits this protein is targeted therapy for CML. This study attempted to identify mutations in genes which can be specific for cervical cancer.

In this study, we amplified specific gene fragments. *EGFR*, *KRAS* and *PIK3CA* genes were selected to be amplified in specific regions since these genes are implicated in most cancers, especially in cervical cancers.

This study shows that Bangladeshi patients have *KRAS* mutation frequency (8.7%) similar to that reported by Wright *et al.* [192]. A 7% mutation in *KRAS* was also reported by Spaans *et al.*, 2015 [193]. Iida *et al.*, [194] also reported somatic mutations in *KRAS* in 3 (6.3%) of 48 cervical adeno/adenosquamous cell carcinomas.

However, surprisingly, *EGFR* mutation frequency is over 6 times higher in our patients (23.91% versus 3.8%) and *PIK3CA* mutation frequency is over 1.6 times (52.17% versus 31.3%) compared to a study by Wright *et al.*, 2013 and 2.6 times higher (52.7% versus 20%) compared to a report [195]. Even *PIK3CA* mutation rate is 3.8 times higher than those in Chinese patients (13.6%) as reported by Xiang *et al.*, 2015 [196].

Survival of patients with *KRAS* mutation is poorer than in patients without *KRAS* mutations [197], therefore, a combination of *KRAS* mutation detection and HPV genotyping would be useful in identifying a patient with poor prognosis for further interventions. Among the three most common histological subtypes of cervical cancer (squamous cell carcinoma (SCC), adenocarcinoma (AC), and adenosquamous carcinoma (ASC) *KRAS* mutations are reported to occur more frequently in AC than SCC [195]. The same study also observed worse disease-free survival (HR 1.57, *P*=0.043) in positive *KRAS* mutation cases. A set of endometrial-like cervical cancers comprised predominantly of HPV-negative tumors and characterized by mutations in *KRAS*, *ARID1A* and *PTEN* was discovered in a study conducted by [198].

EGFR is a membrane tyrosine kinase receptor that is known to contribute to the growth activity and tumor survival, and hence this has become a therapeutic target in several cancers. The extracellular EGFR-binding domain is usually targeted with monoclonal antibodies such as cetuximab or panitumumab, and inhibition of the EGFR tyrosine kinase activity is done with other small molecules as well such as gefitinib and erlotinib, especially patients who have specific functional *EGFR* mutations. In *EGFR* gene, exons 18–21 are the hot spot region for gain-of-function mutations. In previous studies [194, 199] it has been found that a strong correlation between poor prognosis and *EGFR* gene amplification in patients with cervical squamous cell carcinoma. In other carcinomas like leukemia, glioblastoma, and colorectal, gastric, breast, and hepatocellular carcinomas *EGFR* mutation frequency reported to be low. Mutations in exons 19 and 21 of *EGFR* gene were not found in any previous studies [199, 200]. No mutations identified in their samples affecting the EGFR kinase domain in exons 18 through 21 in human neoplastic samples analyzed. However, in cervical carcinoma, we have found a mutation in our Bangladeshi patients. These suggests that mutations in the EGFR kinase domain may be not common in other parts of the world. Our results suggest, therefore, that treatment of CC patients with TKIs needs mutational screening before prescribing drugs and may not have the same efficacy as seen in patients with no-mutation. Therefore, CC patients without such mutation, targeting the *EGFR* with other inhibitors may be more appropriate.

PIK3CA mutations can cause the deregulation of the phosphatidylinositol 3-kinase-Akt signaling pathway, which comprises cell proliferation, transformation, and cell

survival, stimulating oncogenesis. Aberrations in this pathway are described in various cancers, including cervical cancer, and this has led to the development of PI3K-inhibitors and Akt-inhibitors as potential cancer therapies, with some already having reached clinical trials.*PIK3CA* mutation rates are very heterogeneous in different studies (20–37%) [201, 202]. However, patients in our study harbor more mutations (52.17%). In a [195] study, a clear trend was seen for reduced survival in patients carrying a *PIK3CA* mutation, especially with the SCC subtype. Similarly a study [192] also showed that an association lies between *PIK3CA* mutation and shorter survival.

Corresponding amino acid analysis was done to find out the effect of mutations on proteins. Among the 39 different mutations, 17 mutations were located in exon regions. Nine of them were *PIK3CA* mutations and the other 8 were *EGFR* gene mutations. The other 21 mutations were in intron position, hence, they did not have any effect on the amino acid sequence.

The 17 mutations of exon regions were analyzed and it was found out that 15 of them were synonymous. This means that the change in nucleotide base did not change the amino acids produced by their codons. One mutation (A>G) in *EGFR* gene (chromosome 7) at chromosome position 55,170,332 produced a non-synonymous change, that resulted in a methionine to valine substitution at amino acid position 636 of the protein.

We have done different bioinformatics analysis of mutations we have found using tools like Variant Effect Predictor tool, MuPRo tool, Mutation Mapper tool. LoFtool scores of all intronic and exonic mutations which were significantly low, predicts that effect of the mutation on the functionality of the gene is probably damaging. SIFT and polyphen scores showed that effect of the non-synonymous mutation on the function of protein is not that much significant. But the stability of protein may be affected which is predicted by MuPRotool.

To find out the structural changes, mutant protein was modeled and aligned with the wild-type isoform-3 but no structural difference was observed; this mutation may make the protein unstable rather than altering its structure of the functional domain. So we observed a structural change of the protein caused by non-synonymous mutation only in *PIK3CA* gene.

3D structure changing due to the mutation (T>A) observedin *PIK3CA* gene on chromosome 3 position 179,218,237. The T to A base transition has resulted in an amino acid change from leucine to isoleucine at amino acid position 523. This change alters the protein structures in PI3 and PI4-kinases domain. *In vitro* protein level analysis needs to be done to find out the exact effect of this amino acid change.

Persistent infection with high-risk human papillomavirus has been demonstrated to be the necessary causal factor for developing cervical cancer. To know the most prevalent high-risk HPV in different geographical areas is important to design diagnostic tests and implementation of vaccines [203]. Cervical cancer is a highly preventable disease if it is diagnosed at an early stage [204]. Two HPV vaccines are currently on the market: Gardasil (quadrivalent) and Cervarix (bivalent). Both vaccines protect against the two HPV types HPV-16 and HPV-18 [205]. Gardasil gives protection against HPV-6 and HPV-11 also [206]. Protection from HPV vaccine is expected to be long-lasting. But vaccinated women still need cervical cancer screening because the vaccine does not protect against all HPV types that cause cervical cancer [96]. HPV vaccine is routinely recommended for girls 11 and 12 years of age. In Bangladesh, HPV vaccine implementation is required highly. The Global Alliance for Vaccines and Immunization (GAVI) offered HPV vaccine to the developing countries like ours in low price (Lumpur and bdnews24.com). HPV vaccine has been introduced in Bangladesh. It should be affordable to the general population [207].

Fortunately, Merck has recently submitted a Biologics License Application to the US FDA for an investigational nonavalent HPV vaccine, V50330. This nonavalent vaccine appears to be safe and effective in preventing persistent infection and precancerous lesions associated with HPV types -16, -18, -31, -33, -45, -52, and -58, and genital warts related to types -6 and -11 [208].

4.7 Focal points of analysis

Several points are of special importance concerning this study. These are listed below:

- Genotyping of HPV detects the presence of high-risk HPVs (mainly HPV-16 and HPV-18) in 29.5% of asymptomatic healthy female population. They will progress into cancer unless they are treated in this stage.

- Molecular based detection systems reveal presence of high-risk HPVs in the patients even with no cervical abnormalities.
- The most prevalent genotype in apparently healthy population found in this study is HPV-18 which is about 54% alone. In two samples, there were both HPV-16 and HPV-18 was found by multiplex type specific PCR posing a great chance of developing cervical cancer.
- In cancer patients, total 98.26% are caused by associate with HPV among which solely HPV-16 accounts for 46.95% cases while 39.13% patients are infected by both HPV-16 & HPV-18.
- Females of lower socio-economic status are more prone to get infected by HPV. This study figured out the high-risk HPVs are present in 66% of asymptomatic females while 83% of cancer incident with low-income.
- Both the VIA test and molecular detection systems are required for regular cervical cancer screening to detect the cervical cancer.
- As a good number of high-risk HPVs are found in regular, normal patients, there must be a control of transmission of HPV.
- Mutation in *PIK3CA* gene was found to be highest, followed by *EGFR* and *KRAS* mutation.
- Two non-synonymous (missense) mutation were found, one in *EGFR* gene where methionine was changed to valine and another non-synonymous mutation was found in *PIK3CA* gene, where leucine was changed to isoleucine. The affected domain is PI3 and PI4-kinases.

4.8 Concluding remarks

Cervical cancer is a major cause of morbidity and mortality, particularly in developing countries. This study reveals that somatic mutations exist in cancer tissues of cervical cancer patients. Affected women are usually, working, and raising children, which creates substantial social problems.

The data obtained from this study can be used to establish a database for Bangladeshi cervical cancer incidents. Since the findings suggest that cervical cancer may be diagnosed early, we can establish an effective vaccination and treatment plan, and also this cancer might harbor targetable oncogenic mutations, this should encourage further studies to better understand these outcomes and exploit them for clinical use.

New prevention strategies will be implanted very different in each healthcare setting; in a few years, moreover, we are expecting to see a wide variety of cervical cancer prevention programs along with vaccination in parallel.

Nowadays, many cancer diagnoses apply specific mutation detection. Specific mutations for specific cancers are also being exploited for more tailored treatment strategies. Future studies are needed to validate this finding and to explore the biological and clinical importance of these mutations. Precise classification of cervical carcinomas in combination with mutation profiling is valuable for predicting disease outcome and may guide the development and selection of tumor-specific treatment approaches.

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APPENDIX I

Buffers and Reagents

A. Proteinase-K (10 mg/mL)

50 mg of proteinase-K was dissolved in 5 mL TE-buffer.

B. 70% ethanol

70 mL ethanol was added to 30mL distilled water and stored at room temperature.

C. 10x-TBE (Tris-borate –EDTA, pH 8.0)

108 gm of Tris-base, 55 gm of boric acid and 40 mL of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 1000 mL. The buffer was stored at room temperature.

D. 0.5 M EDTA

18.61 gm ofNa2EDTA.2H2O (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 mL of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 mL with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

E. 1x-TE buffer (Tris-EDTA)

TE buffer (10 mMTris-Cl/1mM EDTA, pH 8.0) was prepared was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. For making 1000mL or 1L of TE buffer, 10 mL of 1 M Tris-HCl (pH 8.0) and 2 mL EDTA (0.5 M) and distilled water added up to 1000mL. The buffer was autoclaved and was stored at room temperature.

F. 6X-Gel loading buffer (Glycerol and bromophenol blue)

3mL glycerol (30%) was added to 25mg bromophenol blue (0.25%) and distilled waterwas added upto 10mL.

G. EtBr (0.5 µg/mL)

10 µL of 10 mg/mL Ethidium bromide solution was added to 200 mL distilled water. This solution was stored at room temperature and covered with aluminum foil.

H. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na2HPO4 and 2.0 gm of KH2PO4 in 800 mL distilled water. pH was adjusted to 7.4 with HCL. The final volume was adjusted to 1 L by distilled water.

I. Buffer AW1 (Wash Buffer 1)

19 mL of Buffer AW1 concentrate (provided with the QIAamp® DNA Mini Kit Catalog No.51304) was mixed with 44 mL ethanol (96-100%) before using.

J. Buffer AW2 (Wash Buffer 2)

13 mL of Buffer AW2 concentrate (provided with the QIAamp® DNA Mini Kit Catalog No.51304) was mixed with 43 mL ethanol (96-100%) before using.

K. Buffer B3 (Binding Buffer solution)

For every 21.7 mL B3 (supplied with Invitrogen PCR DNA Purification Kit), 2.3 mL isopropanol (>99%) was added before using.

L. Buffer W1 (Wash buffer)

For every 16 mL Wash Buffer (supplied with Invitrogen PCR DNA Purification Kit, 64 mL ethanol (96-100%) was added before using.

APPENDIX II

Equipments

The important equipment used throughout the study are listed below:

Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Automated thermocycler, Model: 12137	Bio-Rad, USA
Centrifuge, Bench top, Mode1:5804	Eppendorf, Germany
Class II Microbiological safety cabinet	Labcaire, USA
Electric balance, Scout, SC4010	USA
Freezer (-30°C)	Liebherr, Germany
Gel documentation	Sigma, USA
Horizontal gel electrophoresis apparatus Hl-SET	UK
Incubator	Japan
Microcentrifuge, Mikro20	Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
pH meter, Model no: MP220	Eppendorf, Germany
Power pack	Toledo, Germany
Refrigerator (4°C)	Vest frost
Room temperature horizontal shaker	Gerhardt, Germany
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model: SUM	England

APPENDIX III

CONSENT FORMS

Consent form for Specimen (tissue and blood) and Data collection for research purpose

Study title: Genotype Distribution and Sero-epidemiology of Human Papillomavirus (HPV) in Female Population of Bangladesh

INVESTIGATOR:

Dr. Mahmuda Yasmin, Principal Investigator,
Professor, Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh.

PURPOSE:

As cervical cancer is prevalent in Bangladesh, mortality rate is high in the context of low socioeconomic condition, we are experiencing an increasing burden of this disease; so there is indication to conduct genetic research in our country. We will perform molecular profiling including HPV detection and genotyping testing, mutation detection, association of *C. trachomatis* with HPV and blood protein marker detection**.** Outcome of our result can help clinicians in prevention and early diagnosis, understanding of prognosis & intervention in treatment modalities of this cancer.

RISKS AND DISCOMFORTS:

Donating blood or specimens for research may have little risk. Researchers believe the chance of this risk is very small and protections are in place to lessen this risk. Even then, during biopsy, patients may feel some pain. Some unwanted complications may arise while collecting the tissue samples. All personal medical information about donor and any information obtained from this study of donor's specimen will be preserved in a secured database. Donor's name and identity will be used only for data collection and will not be disclosed to a third party. All reasonable efforts will be made to protect the confidentiality of information that can in any way be connected to donor.

BENEFITS:

Taking part in this research will not benefit to donor directly; however, what we learn may help others in the future.

ALTERNATIVES:

Donor may choose not to take part in this study.

COSTS:

Donor will bear his/her own treatment expenditure. There will be no cost to donor for any procedures required for the research. Donor will not be paid or given any other award for taking part.

PROPERTY DONATION:

By agreeing to take part, you allow the use of your samples for the research described in the purpose section of this document. In addition, you agree that we may make any lawful use of your samples, including future research studies, destroying them, or transferring them to a public or private entity.

CONFIDENTIALITY:

Every effort will be made to keep donor's information records private. All others, including employers, insurance companies, personal physicians, and relatives will be refused access to the information and to the samples, unless you provide written permission, or unless we are required by law to do so. Anything that can identify you will be kept in private, protected files. An ID number will be assigned to you, your tissue samples, and information about your medical history. Only the investigator named on this consent form will be authorized to link the ID number to your name. The link of your ID number to your name or any other identifying data will be stored in the established secure database. Any future research done on any of the samples must be designed in a way that protects your privacy and presents research results and data anonymously. The Institutional Review Board must also monitor it.

SPONSORSHIP:

The possible funding agencies for this study are Ministry of Science and Technology, Govt. of Bangladesh and University Grant Commission of Bangladesh.

CONFLICT OF INTEREST:

The principle investigator or the co-investigators and the other possible research team members have no conflict of interest to declare.

PARTICIPATION:

Shahana Sharmin, cell phone: xxxxxxxxxxx has offered to answer any other questions donors may have about this study. If donor has any questions regarding his/her rights as a research subject, he/she may contact us. If in the future donor decide no longer to take part in this study, we will destroy all identifying information and will not use your tissue in any future research. However, donor's tissue samples are already being used in current research project and if their withdrawal jeopardizes the success of the entire project, we may continue to use them until the project is completed. Donor will be given a copy of this consent form for his/her records. Donor's signature below indicates that donor has red this consent form and agree to take part in this study.

CONSENT:

By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of taking part in this study. I understand that I have the right to refuse to take part in the study. My decision about taking part in the study will not affect my health care.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my medical records will be kept private except as described to me.
6) I understand that no information about me will be given to anyone or be published without first asking my permission.
7) I hereby knowingly and voluntarily authorize you to use my specimen and my Protected Health Information in the manner described in this Consent Form.
8) I agree, or consent, that I may take part in this study.

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Date Subject's Signature

Witness-1:

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Date Signature

Witness-2:

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Date Signature

APPENDIX IV

DATA COLLECTION SHEET **Date:**

Project Title: Genotype Distribution and Sero-epidemiology of Human Papillomavirus (HPV) in Female Population of Bangladesh

ID		Urban	Rural	Name		Cell	

Age		Marital status	M	UM	Education				
					Illiterate	Literate	Secondary	H. secondary	Above

Address		Monthly income	Tk	Sanitation	

Menorrhagia		Postcoital Bleeding		Postmenopausal Bleeding		Intermenstrual Bleeding		Continued Bleeding	
Y	N	Y	N	Y	N	Y	N	Y	N

Pain		Weight Loss		Menarche	
Y	N	Y	N		

M	F	Age at time of marriage		AL C		P		G		1st PG	Yam	2nd PG	Yam	3rd PG	Yam

Smoking		OCP		Injection HC		Betel nut		F/H		Hb	ESR
Y	N	Y	N	Y	N	Y	N	Y	N	gm/dl	Mm

TC	cmm	N	%	L	%	LFT		Creat	mg/dl	RBS	mg/dL

Histo pathology	SCC	Adeno carcinoma	Other:	Informed about HPV vaccine	HPV vaccinated	Y	N

Staging		Informed about cervical carcinoma	Y	N

Treatment	Surgery	chemotherapy	Radiotherapy	Recurrence	Y	N

Comment	Signature of Investigator: