Molecular Characterization of

HPV-16 L1 gene

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List of Abbreviations

µm	Micro meter
AA	Amino acids
AD	Adenocarcinoma
Bp	Base pairs
CIN	Cervical intraepithelial neoplasia
dsDNA	Double stranded DNA
E	Early
FFPE	Formalin fixed paraffin embedded
Gel-Doc	Gel documentation system
HPV	Human Papillomavirus
ICC	Invasive cell carcinoma
Kb	Kilo base pairs
KDa	Kilo Dalton

L	Late
M	Molar
mM	Micro molar
PCR	Polymerase Chain Reaction
RPM	Revolutions Per Minute.
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial neoplasia
STV	Sexually transmitted virus
TAE	Tris-Acetate-EDTA
µl	Micro liter

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Abstract

Human papillomavirus (HPV) is the most common cause of sexually transmitted infections in the world. HPV is responsible for the development of virtually all cervical cancer cases. In addition to that there is mounting evidence showing its possible involvement in cancers other than cervix. The currently available prophylactic vaccines are based on the self-assembling property of the major capsid protein encoded by L1 gene. The high cost associated with these vaccines prevents their use in most developing countries. Therefore, the current aims of the study involved HPV screening of various paraffin embedded cancer samples, followed by molecular characterization of L1 gene with an aim to predict epitopes for the future development of subunit vaccine in Pakistan. These objectives were achieved by using PCR and in-silico approaches. General primers (GP) and type specific primers (TS16, TS18) were used to amplify a conserved region in L1 and E6 gene. The sensitivity of type specific primers was found to be better than the GP. With the use of GP, HPV was detected in about 50% cases while TS primers revealed 75% of HPV positive cases. Single infection of HPV16 involved in 64.5% cases and about 27.7 % of cases positive for HPV-18 and in 21% of cases co-infections of both types of HPV. A strong association of HPV-16 with cancers other than that of cervix was also found. The amplification of L1 gene particularly from the formalin fixed paraffin sections (FFPE) have been challenging, however the limited number of samples exhibited

conservedness. In view of high cost of currently available vaccines, the in-silico approach was used for designing a cost effective subunit vaccine. With this approach, 10 B-cell epitopes were identified with four epitopes showing more than 90% conservedness. Five of the tolal 35 T-cell epitopes showed more than 80% conservedness. These epitopes can be potential targets for the future generation of a cost effective vaccine.

CHAPTER 1

INTRODUCTION

Cervical cancer, the cancer of cervix arose when unusual growth of cells occur in cervix. Cervical cancer is among the seventh most common cancer globally in women (Stewart & Wild, 2014) with the prediction of annually 528,000 new cases and reported 266,000 deaths in 2012 which is responsible for 7.5% of all female cancer deaths (Ferlay et al., 2015). In the developing countries due to lack of screening programs cervical cancer is the fourth most common cancer in women which affects 5,00,000 women per year accounting for 80% of all cervical cancer incidence.

Pakistan comes under the top ten countries which are affected with cervical cancer but according to an estimate incidence and death rate of cervical cancer is low as compared to other developing countries with an estimation of 59.04 million women who are at risk of developing cancer, among them 2,876 women died with this cancer each year (Ferlay et al., 2010b). Like all other cancer rate of cervical cancer higher among developing countries than developed countries as shown in figure 1.1(Ferlay et al., 2010a).

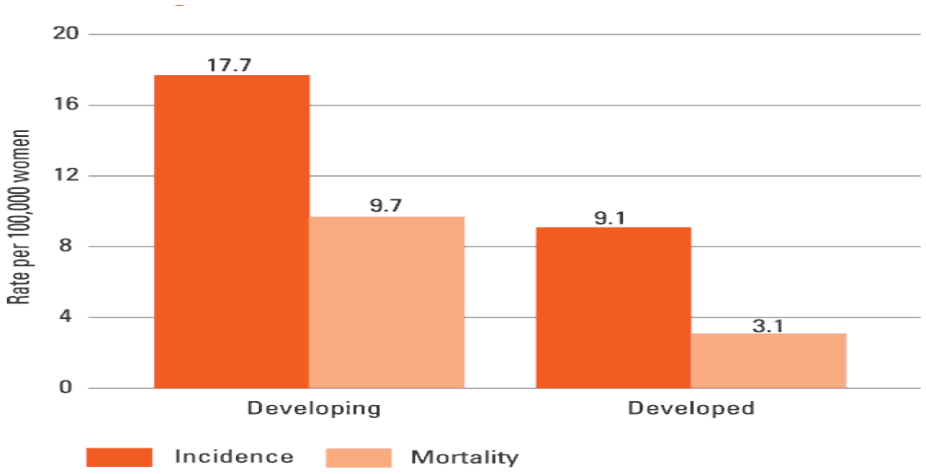

Figure 1.1: rate of cervical cancer among developed and developing countries

Source: (Ferlay et al., 2010a)

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About 70-80 % of cervical cancer cause squamous cell carcinoma which affect the ectocervix, the most prevalent type of cervical cancer than adenocarcinoma which accounts for only 10% of cervical cancer, but recent reports showed increasing incidence of adenocarcinoma. The condition of cancer becomes worsen when these two types combine and causes adenosquamous carcinoma.

In the early stage of cervical cancer it is difficult to diagnose as they don't give any sign or symptoms until the cancer progress towards advance stage. In addition to that it is shown that vaginal discharge with odour, pain during sex and vaginal bleeding after sex is among the most common symptoms. When the cancer move towards advance stage it gives symptoms like constipation, bloody urine, urination problem, back pain , swelling, loss of appetite, weight loss, and fatigue.

When cervical cancer is in the early stage it can be easily curable but as this cancer metastasizes to other parts of body the condition becomes more worsen and difficult to treat. Cervical cancer divides in to four stages on the basis of metastasis and hence stage IV is more complex as compared to initial stages, radiation only help to cure early stages of cancer but as the disease advances, radiation will be inadequate to eradicate the cancer and will also require chemotherapy (Averette et al., 1975).

In order to remain healthy and safe women especially in developing countries need to screen herself. In consistent with this a lot of studies showed that chances of cancers low if screening programs were in use by the community (Moyer, 2012).

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For screening purposes, usually pap smear test is used which allows the detection of any abnormal growth in cervix. If any abnormal changes detected in pap smear test the doctors then move towards further tests or biopsy taken to confirm the disease state (Carpenter & Davey, 1999). Some studies showed that if cancer diagnosed and treated at initial stages the success rate of cure goes above 80% and survival rate also increases. But due to lack of awareness and unavailability of screening programs, the incidence of cancer remains alarming as compared to developed countries where better screening methods are in place.

Papillomaviruses belongs to the family of the papillomaovaviridae. HPV is relatively small non enveloped virus containing circular DNA of 8000 base-pairs. With reference to current research, more than 200 different types of HPV have been identified among these about 40 types comes under genital HPV that attack the genital organs (De Villiers et al., 2004). HPV genome consists of three regions. The first region called long control region (LCR) consists of non coding region. The second region called as early region which include ORFs, E1, E2, E4, E5, E6, and E7. The third region contains L1 and L2 proteins which encodes viral capsid proteins also called as late region (Fields et al., 1996).

The life cycle of papillomavirus is unique from all other types of viruses because in their life cycle the infection need epidermal or mucosal epithelial cells. In these cells partial expression of E5, E6 and E7 enhanced the proliferation of the infected cells.

Set of studies showed that in addition to HPV infection which accounts for almost all cervical cancers, many factors including western life style, pregnancy and sex at younger age, sexual behaviour of woman's partner, use of oral contraceptives and Diethylstilbestrol, smoking, overweight, low diet, poverty and weakened immune system contribute towards cancer (Waggoner, 2003).

For the first time in 1949 electron microscope was used for the detection of human papillomavirus and in 1980 the association of HPV in cervical cancer was first reported (Franco, 1995). In 1996, the World Health Association and European Research Organization on Genital Infection and Neoplasia and the National Institutes of Health Consensus Conference on cervical cancer accepted HPV as main reason of cervical cancer. In the past group of studies and international review parties strongly correlates that Human papillomaviruse shows strong association with cervical cancer (Harro et al., 2001)

In 2004 research was carried out in order to check the distribution of different types of HPV in cervical cancer worldwide and they found that HPV-16 and 18 are constantly involved in cancer of cervix. From these findings it was concluded that HPV is associated in almost all cervical cancers with the highest prevalence of high risk types HPV-16 and 18 (Munoz et al., 2004).

Human papillomavirus is responsible for about 5% of all cancers globally and hence conflicting data available which shows the involvement of HPV in different cancers.

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High risk HPV-16 and 18 shows strong relation in the development of various anogenital and non-anogenital cancers. The studies on the notorious role of HPV in other anogenital cancers, and non anogenital such as breast cancer and lungs cancer is inadequate but the obvious epidemiological studies indicate that these cancers can be caused by HPV.

Lungs cancer is leading cause of death worldwide both in males and females. Main aetiological factor responsible for lungs cancer is controversial. Human papillomavirus (HPV), known to be an etiological agent for genital cancers, has also been suggested to be a possible contributory agent for lungs cancer (Prabhu et al., 2012). Different studies performed in order to investigate the role of HPV in lungs cancer and concluded that HPV is second leading cause of lungs cancer after smoking (Klein et al., 2009; Roglic et al ., 1974).

High risk HPV has been recognized as being causal in almost all cervical cancers. In recent years, evidence indicates that HPV may also have a role in breast cancer. The involvement of the virus in breast cancer remains questionable. Earlier reports have shown that HPV DNA was detected in breast cancer specimens and the prevalence of HPV positive breast cancer vary from one study to another. High risk types 16, 18 and 33 of HPV have been screened in breast cancer patients from a wide group of different populations using different techniques. However, the etiological role of HPV in BC development is not clear (Lawson et al., 2006).

The role of human papillomavirus in prostate cancer is controversial as alot of studies carried out to explore the role of HPV in prostate cancer but concluded with opposing results (Korodi et al., 2005).

Ovarian tumor is a common neoplasm of the female ovaries is the most frequent cause of death from gynecological malignancies (Platz & Benda, 1995). In 1987 for the first time ovarian tumors analyzed for HPV prevalence by Kaufman and his fellows, they analyzed a series of patients with advanced ovarian adenocarcinoma and shows positive association in almost all cases. In 1994 another study was proposed by same group of people their study confirm that HPV-16 were detected in more proportion as compared to HPV-18 (Lai et al., 1994).

The aetiologic role of human papillomavirus (HPV) in a variety of malignancies of cervix is well established now the presence of HPV in endometrium has also been reported, although less commonly. In a group of study Fujita and his coworkers investigated the role of HPV in endometroid carcinoma by using different techniques such as PCR, in situ hybridization and southern blotting in a large group of sample from different regions, they find out positive association of HPV in endometroid cancer (Fujita et al., 1995).

Rectal cancer is the growth of abnormal cancerous cells in the lower part of the colon. Actual cause of the cancer is unknown, but risk factors include increasing age, family history, high-fats, smoking or a history of polyps but now in recent years controversial role of HPV in rectal cancer is increasing but defined results are still undergoing. For this

purpose a lot of research should be conducted in order to determine if a strong relationship exists between HPV infection and rectal cancer.

This alarming number of cases requires effective treatment and preventive vaccine so as to eradicate the cancer from the community. In the past two commercially available prophylactic vaccines designed against HPV infection called as Gardasil and Cervarix (Villa et al., 2006).

These HPV vaccines targeting HPV16 and HPV18 have the potential to produce a dramatic drop in cervical cancer rates if they are widely used. It is important to note that over 80% of cervical cancer cases occur in developing nations. This reflects the lack of cytological screening programs and intervention in these countries. In these settings, therefore, the cost of HPV vaccination must be borne in addition to that of screening. Thus, it would be highly desirable to develop a more affordable vaccine for the developing world. In low-resource settings, peptide subunit vaccines against HPV infections have clearly the potential to reduce incidence of cervical cancer cost effectively.

In view of these limitations there is utmost need of peptide subunit vaccine that meet the demands of current situations. For this Novel vaccines should be designed on the basis of molecular technology for eliciting proper immune response against HPV, including both broadly neutralizing antibodies and effective T-cell response. The concept of vaccines is based on identification and chemical synthesis of B-cell and T-cell epitopes which are immunodominant and can induce specific immune responses.

The epitope is recognizable by the immune system part of the antigen, and in particular by antibodies, B cells or T cells. The epitopes may belong to both foreign and self proteins, and they can be categorized as conformational or linear, depending on their structure and integration with the paratope. T-cell epitopes are presented on the surface of an antigen-presenting cell (APC).

Immunogenic epitopes used as peptide vaccines or polytope DNA vaccine are promising approach for HPV with high mutation rates. However, appropriate design and primary in silico analysis is an essential prerequisite before commencing costly transgenic animal studies.

CHAPTER 2

LITERATURE REVIEW

Cancer is among the prominent cause of mortality in developed countries whereas the second leading cause of death in developing countries (Ferlay et al., 2010b)

2.1 Cervical Cancer

Cancer arises when cells of the body grows beyond any control. Cancer is always named for the part of the body where it starts, however it spreads to other body parts later. Cervical cancer is cancer arising from cervix. It is due to the abnormal growth that have the ability to invade or spread to other parts of the body (Jemal et al., 2011).

2.1.1 Anatomy of cervix

The cervix belongs to the female reproductive part. The length of cervix is about 2-3 centimetres and it is situated at the lower, narrow end of the uterus as shown in Figure 2.1. Cervix act as a connecting point between the vagina and upper part of the uterus. The lower end of the cervix bulges through the the vagina called as ectocervix and the cervical opening is called the external orifice.

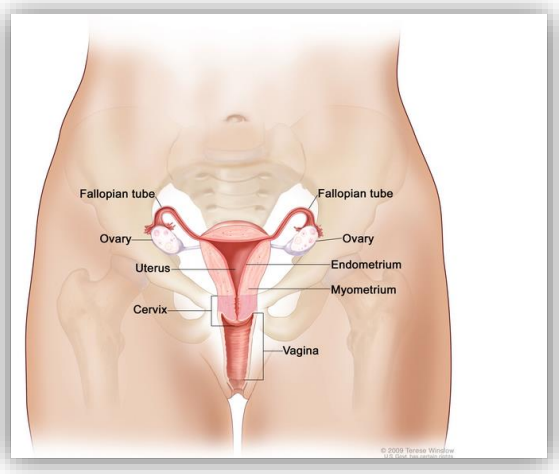


Figure 2.1: Anatomy of uterine cervix

The ectocervix has a convex, egg-shaped surface

and is separated into anterior and posterior lips. The size and shape of the external opening and the ectocervix depends on the age, hormonal state and the process of childbirth.

Blood is supplied to the cervix by artery of uterus. Nerves of cervix pass on the feeling of pain from the cervix to the brain. Presence of HPV merely does not lead to cervical cancer always because in some cases due to cell mediated immunity around 90% of HPV-infected women clear the virus within two years. Studies also reported that whether a woman will develop cervical cancer depends on a variety of additional factors that act in connection with cancer-associated HPV types. However, it remains unclear which type of immune cells are implicated in this process (Amador et al., 2013).

2.1.2 Prevalence of Cervical Cancer

Cervical cancer the cancer of cervix is the fourth most common cancer in women, and the seventh overall, with an estimated 528,000 new cases reported in 2012. There were an estimated 266,000 deaths from cervical cancer worldwide in 2012, accounting for 7.5% of all female cancer deaths. Almost nine out of ten (87%) cervical cancer deaths occur in the less developed regions (Ferlay et al., 2015).

Cervical cancer is one of the most frequent cause of cancer in women in developing countries. Probably, cervical cancer affects 500,000 women each year. Almost all cervical cancer results from genital infection with human papillomavirus (Bosch & De

Sanjose, 2002). According to Information center on HPV and cancer (ICO) Pakistan has a population of 59.04 million women who are at risk of developing cervical cancer but still there is a lack of accurate data on incidence and mortality rate of Cervical Cancer in Pakistani isolates.

Like all other cancers rate of cervical cancer is higher in developing countries. Countries with higher incidence include India, Africa and Melanesia and lowest incidence in Australia and New Zealand (Ferlay et al., 2010b).

The figure 2.2 shows the high prevalence of HPV in developing countries. India shares the highest burden of cervical cancer globally. In 2008 out of 132000 cases of cervical

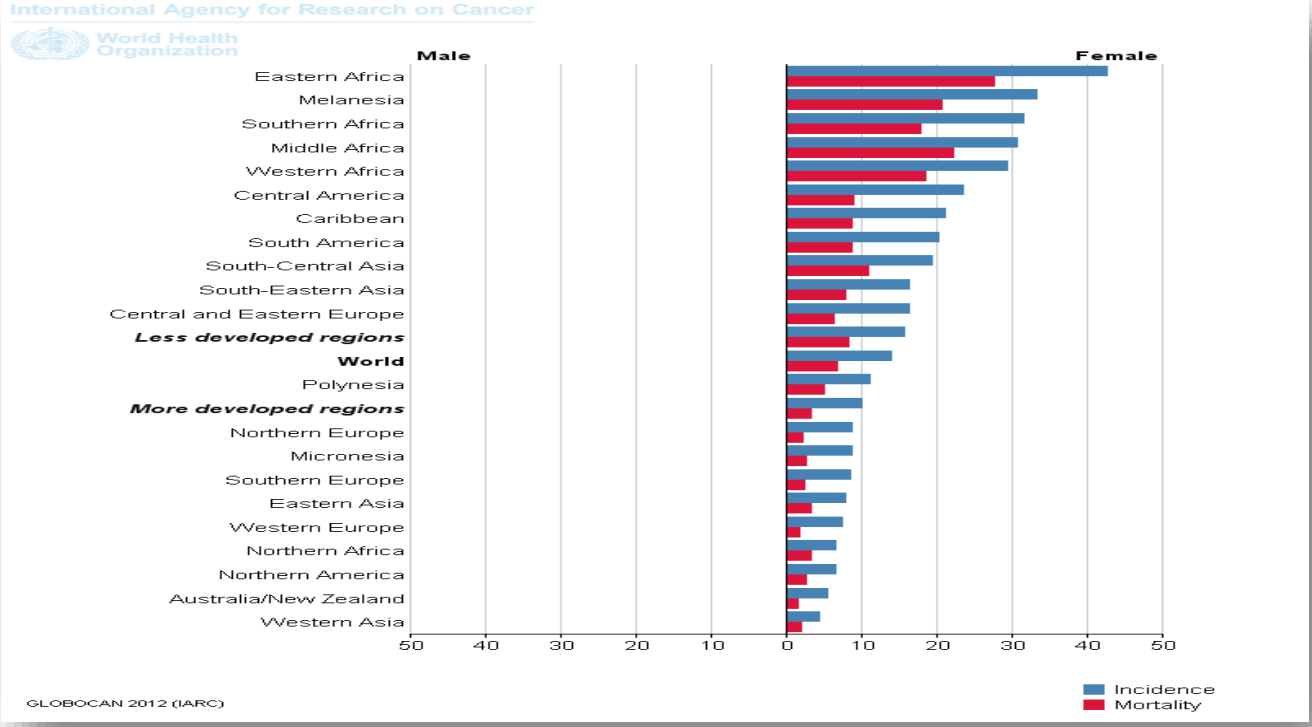


Figure 2.2: Incidence and mortality rate of cervical cancers in different countries

Source: GLOBOCAN 2012

cancer about 74000 deaths occurred (Basu et al., 2009).In 2008, Africa was reported to have about 667,000 cases of cancers, out of which cervical cancer was the most prevalent one. The high mortality rate is because of the poor hygienic and medical facilities (Denny & Anorlu, 2012).

Histologically cervical cancer categorized in to different subtypes.

Squamous cells are a type of a flat, skin like cells that wrap the outer surface of the cervix (the ectocervix). About 7 to 8 out of 10 cases of cervical cancers are squamous cell as shown in figure 2.3.

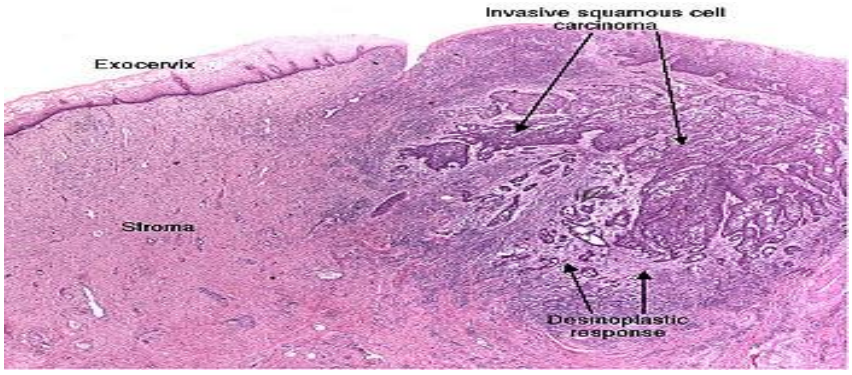


Figure 2.3 showing invasive squamous cell carcinoma

Source: (Tunio et al., 2015)

- Adenomatous cells are gland cells that makes the mucus. The cervix has these gland cells scattered along the inside of the passageway that runs from the cervix to the womb (the endocervical canal) as shown in figure 2.4. Adenocarcinoma is a cancer of these gland cells. It is less common as compared to squamous cell cancer, but has become more common in recent years.

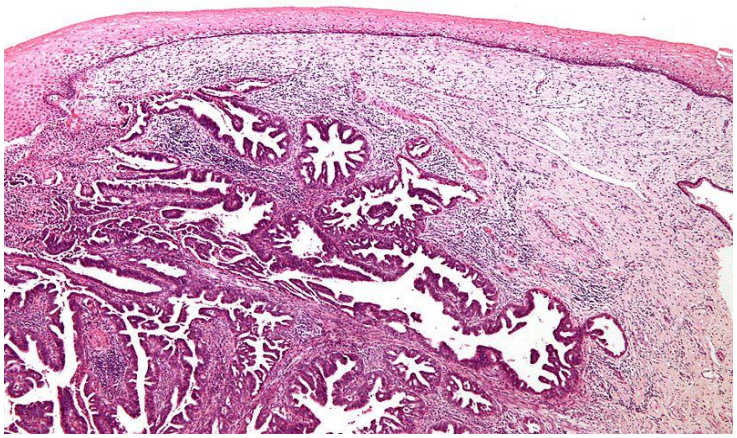


Figure 2.4 Showing Adenocarcinoma of cervix

Source: (Hopkins et al., 1988)

Adenosquamous carcinoma is the most worsen type of cancer as it contains the properties of both adenocarcinoma and squamous carcinoma. Other rarer types of cervical cancer include Leiomyosarcoma of the cervix, small cell carcinoma, glassy cell carcinoma.

2.1.4 Stages of Cervical Cancer

Cervical cancer is staged by the International Federation of Gynaecology and Obstetrics (Pecorelli, 2009) staging system, which is based on clinical examination, rather than surgical findings. Diagnosis of cervical cancer that helps to define the different stages can be classified by the use of the histopathological criteria which follows the royal college of pathologists. The reports of the cervical tumors indicate the type of tumor, size and extent of the tumor. Also the depth and pattern of invasion could be determined. This assessment should be thoroughly done and well standardized since the diagnosis determines the start and path of treatment (James et al., 2008) as shown in figure 2.5.

Cervical cancer is staged using the Tumor-Node-Metastasis (TNM) system in to 4 stages. In Stage 0 cervical cancer the cancer cells are confined to the surface of the cervix. This stage is also called carcinoma in situ (CIS) or cervical intraepithelial neoplasia (CIN) grade III.

In Stage 1 the cancer has grown deeper into the cervix, but has not spread beyond it. This stage is further separated into two subcategories on the basis of size of tumor. surgery or radiotherapy used to treat stage 1 cervical cancer, however further advanced stages such as 1B2 require combined chemotherapy and radiotherapy. Stage II, defines the stage at which the cervical cancer cells grows beyond the cervix and uterus, but have not yet reached the lower part of the vagina and walls of the pelvis. In this stage the disease has not spread to lymph nodes or distant sites. Stage II further divides in to subcategories on the basis of tumor size.

In Stage III, the cervical cancer, cancer spread away from the cervix into the surrounding structural area of pelvic. It may have grown down into the lower part of the vagina and the muscles and ligaments that line the pelvis. It may have grown up to block the tubes that drain the kidneys. It can be divided into further subtypes depends on metastasis.

In Stage IV the cancer has spread to other body organs outside the cervix and womb. Further categorized in to subtypes on the basis of metastasis means in 4A stage the cancer has spread to nearby organs whereas in 4B cancer has spread to organs away from cervix like lungs etc. These stages of cancer may be treated with combinations (Averette et al., 1975).

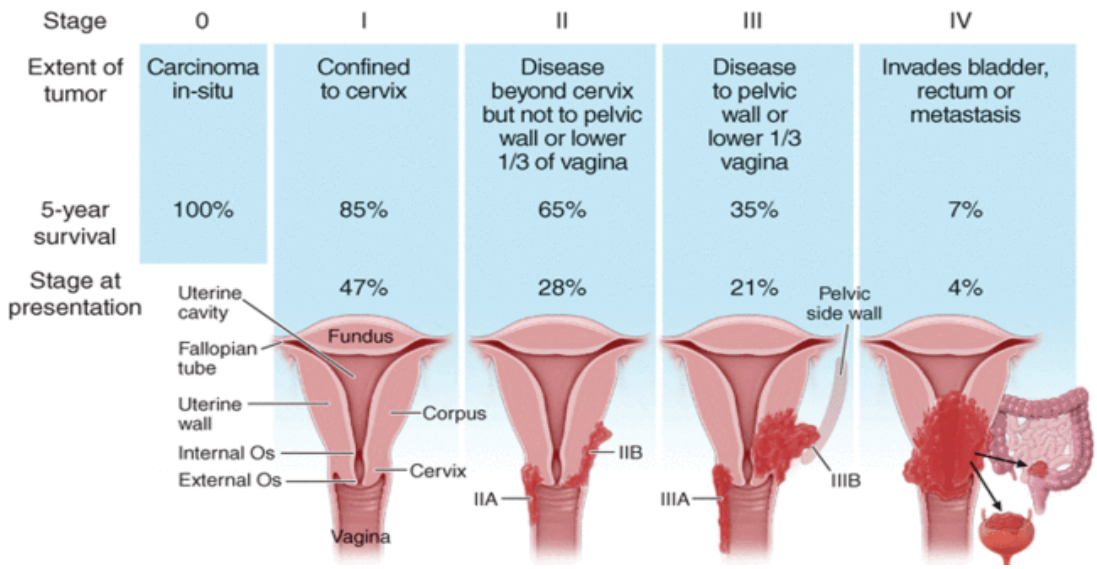


Figure 2.5 Stages of Cervical Cancer

Source: (Averette et al., 1975)

2.1.5 Risk Factors

A risk factor is anything that increases the risk of developing cancer. For every cancer there are different risk factors. One of the most important factor associated with cervical cancer is infection with HPV accounting for two-thirds of all cervical cancer because there are about 40 to 50 types of HPV responsible for cervical cancer in women but about 5% of women infected with HPV go on to develop cancer (Zur Hausen, 2002). Most men and women who are sexually active have been exposed to HPV at some time in their lives, but most infections clear up on their own (Bosch et al., 1996). Some types of HPV can cause changes in the cells of the cervix, if these changes were detected at early stage they were treated easily (Bosch et al., 1995). Another important factor

responsible for cervical cancer is the link between age and pregnancy. Any woman who ever had sex and have many pregnancies are at risk of developing cancer but the chances are higher for women who had their first pregnancy and sex at very early age as compared to women who had their first pregnancy at the age of 25 or above (Munoz et al., 2002).

Another great risk of cervical cancer depends on the sexual behaviour of woman's partner. Those women having multiple sexual partners puts her at higher risk of acquiring the HPV infection (Kamau, 2011). In addition to that there is a great risk associated with cervical cancer is the use of oral contraceptives, women who use contraceptives for long time are at higher risk of cervical cancer but the risk of developing cancer goes down as they no longer take the contraceptives (Piper, 1985). In the past a great risk associated with cervical cancer is the use of hormonal drug Diethylstilbestrol, women whose mother use this drug to prevent miscarriage were at higher risk but now this risk factor is no longer effective as FDA stop the use of this drug during pregnancy in 1971 (Moyer, 2012). Having a family history of cervical cancer is also main risk factor of cervical cancer as cervical cancer run in families, women are at higher risk of cervical cancer whose family members affected with this cancer. Other risk factors include smoking, being overweight, low diet, poverty and weakened immune system (Waggoner, 2003).

2.1.6 Signs and symptoms

In the early stage cervical cancer may or may not give any sign and symptoms until the cancer progress towards advance stage and sometimes the symptoms resembles to the symptoms of genital Chlamydia trachomatis infection. In most cases pain during sexual intercourse, vaginal bleeding after sex and vaginal discharge with odour and may be pale, watery, pink, brown, bloody or foul smelling is among the most prominent symptoms of cervical cancer. In advanced stage of cancer when the cancer metastasize to other organs of the body it provoke other symptoms like bloody urine, uncontrolled urination, constipation, pain in bones and back, swelling of legs and kidney, loss of appetite, weight loss, tiredness and a lack of energy.

2.1.7 Diagnosis

To remain safe and healthy women needs to screen herself regularly if any of sign or symptoms appear then the women should have to go for check-up. Routine cervical screening detects abnormal cervical cells before they have a chance to turn in to cancer.

Cervical cancer is a disease that develops quite slowly and begins with a pre-cancerous condition known as dysplasia. Dysplasia is easily detected in a routine smear and is completely treatable. Detecting and treating abnormal cervical cells early can almost prevent cervical cancer from developing. Between 60% and 80% of women diagnosed with cervical cancer had not had a smear test within 5 years of their diagnosis (Moyer, 2012).

The prognosis is better when the cancer is found at very early stage (Moyer, 2012) . Diagnosis of cervical cancer can be done in many ways but firstly comes physical exam of the body to check any unwanted growth or lumps. After physical exam if something unwanted growth appear then consultants move towards pelvic exam in which genital areas of women checked for any abnormal growth.

To visualize abnormal growth under microscope a pap test is generally done to take cells from vagina or cervix using speculum (Carpenter & Davey, 1999)

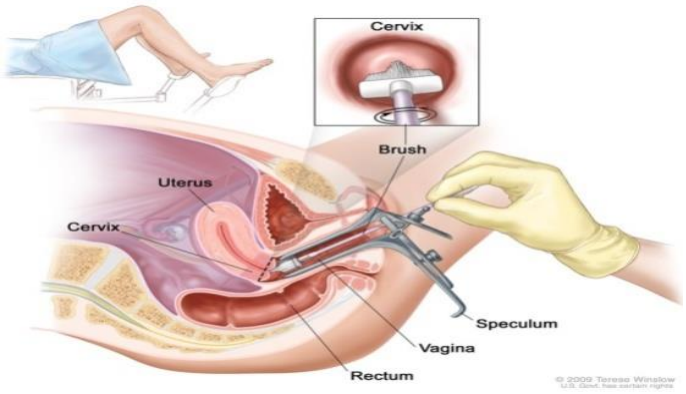


Figure 2.6 Pap test

Source : Carpenter & Davey, 1999)

If any abnormal findings comes after pap test then cells obtained from pap test used to check for HPV DNA and doctor can go for biopsy in which sample of tissue is cut from the cervix and viewed under a microscope.

Different methods employed to take portion from cervix in order to investigate the cervix such as punch biopsy, colposcope, endo-cervical curettage and conization.

Punch biopsy is a procedure in which doctor uses a sharp tool to pinch a small section of cervical tissue. Colposcope uses magnifying lens which make it easier to see the cervix (Stafl & Mattingly, 1973) as shown in figure 2.7

In endo-cervical curettage doctor uses a spoon shaped instrument to scrape a small section of cervical tissue (Andersen et al., 1988). Conization also called as cone biopsy in this procedure doctors removes cone shaped section of tissue from abnormal cervix (Nagai et al., 2000).

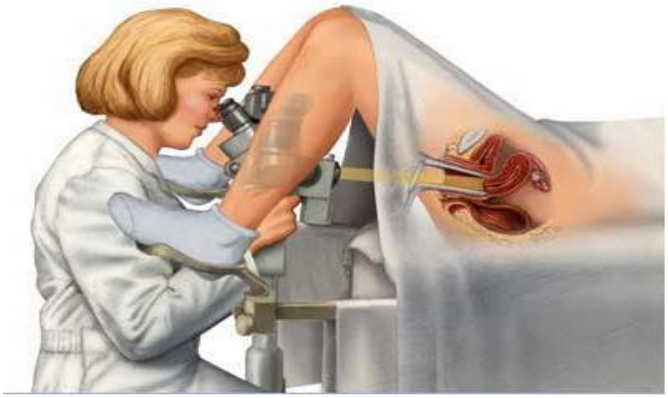


Figure 2.7: Colposcopy

Source: (Stafl & Mattingly, 1973)

2.1.8 Treatments for Cervical Cancer

If cervical cancer detected at early stage and proceeded towards treatment then chances of patient's survival increases. It was estimated that around 80% of cases cured when diagnosed and treated at early stage. But the fact is that, in the developing countries cervical cancer frequently diagnosed at very late stage due to unavailability of good

screening methods as compared to the developed countries where cancer can be diagnosed at early stage and hence cured by using effective treatment. The success of screening and treatment in developed countries also depends on females who voluntarily participate in screening programs (Gustafsson et al., 1997). The treatment of cervical cancer depends on the stage of cancer in order to give the best treatment stage of the disease needs to be identified (Waggoner, 2003). Staging is a careful attempt to find out whether the tumor has invaded nearby tissues or not. When cancer metastasizes to other parts of the body the new tumor has the same kind of cancer cells and name of the cancer remains same. If cervical cancer spread to the lungs, the cancer cells in the lungs are actually cervical cancer cells and are known as metastatic cervical cancer. For this new tumor is called as distant or metastatic disease.

Treatment options for cervical cancer are surgery, radiation therapy, chemotherapy or a combination of methods. The choice of treatment depends mainly on the size of the tumor and whether the cancer has spread. Cancer treatments often damage healthy cells and tissues, so side effects are common. Side effects may not be the same for each person.

In the past early stage of cervical cancer was cured with radiotherapy or radical hysterectomy with success rate of 80%. Early stage of cervical cancer treated by using surgery or radiotherapy both. In addition to that radiation therapy is used with any stage of cancer. National Cancer Institution based on their research supported that advance

stage of cancer can be cured effectively if radiotherapy combine with chemo radiation (Thomas, 1999).

Two types of radiation therapy given to cancer patients Internal radiation therapy and External radiation therapy. In internal radiation therapy a thin tube loaded with radioactive substance is placed inside the vagina. When tube is removed, no radioactivity is left in the body. It can be repeated two or more times over several weeks.

In external radiation therapy large machine directs radiation at the pelvis or other tissues where the cancer has spread. External radiation usually takes place 5 days a week for several weeks. It is similar to an x-ray.

Developing countries use diverse types of treatments such as cryotherapy and Loop electrosurgical excision procedure (LEEP). Cryotherapy is cheap and more affordable method as compared to Loop electrosurgical excision procedure (LEEP).

To treat the cervical cancer at advance stage one of the most important treatment is intracavitarybrachy therapy where radioactive sources are used and focus near to the targeted tumor. By this method tumor decline immediately and thus eliminate the tumor growth (Long et al 2007). Thirty-five percent of women with invasive cervical cancer have persistent or recurrent disease after treatment.

As cervical cancer is a type of recurrent cancer means the disease sometimes returns because undetected cancer cells remained somewhere in the body after treatment so regular checkups after treatment is important. Checkups help ensure that any changes in

health are noted and treated. Checkups include a physical exam, cervical smear tests and chest x-rays.

2.1.9 Prognosis of Cervical Cancer

Prognosis depends on several factors like age, type of cancer and stage of the cancer among them stage of cancer is more important. There is increase of survival rate in patients whose cancer diagnosed at early stage. In early stage of treatment survival rate of the 5-year is 92%, and thus survival rate decreasing as the cancer progress towards the advance stages As shown in the table 2.1

Stage	5-Year Observed Survival Rate
0	93%
IA	93%
IB	80%
IIA	63%
IIB	58%
IIIA	35%
IIIB	32%
IVA	16%
IVB	15%

Table 2.1: Survival rate after treatment

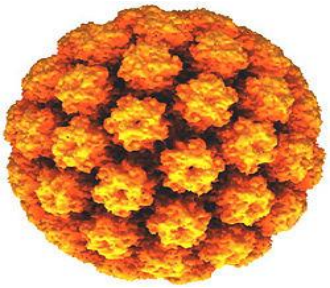
According to the International Federation of Gynecology and Obstetrics, survival improves when radiotherapy is combined with cisplatin-based chemotherapy (Green et al., 2001).

As the cancer metastasizes to other parts of the body, prognosis drops dramatically because treatment of local lesions is generally more effective than whole body treatments such as chemotherapy (Weir et al., 2003). Regular two-yearly Pap tests can reduce the incidence of cervical cancer by up to 90% and save women dying from the disease each year.

2.2 Aeteiological agent of Cervical Cancer

Human papillomavirus (HPV) are the principal etiologic agents for both cervical cancer and its precursors. Human papillomavirus (HPV) is amongst the most predominant reasons for sexually transmitted viral diseases around the world. HPV continues to be a promising topic because of increasing infections continuously (Bosch & Munoz, 2002).

2.2.1 Structure of Human Papillomavirus 16



Papillomaviruses belongs to the family of the papilomaovaviridae. HPV-16 belongs to alpha-papillomavirus genus and is related to squamous and adenocarcinomas of cervix,. Fifteen percent of this genus is responsible for invasive cervical cancers (ICC). HPV-16 has icosahedral capsid made up of seventy two capsomeres that are present around the genome (De Villiers et al., 2004). Structurally HPV is quite small non enveloped virus with a diameter of 55 nm. When viewed by electron microscopy virus looks like a golf ball. Human papilomavirus is double helix circular DNA made up of 8000 base-pairs. With reference to current research and on the basis of DNA sequence more than 200 different types of HPV have been identified (De Villiers et al., 2004). Out of these about 40 types attack the genital organs.

The genome of HPV is functionally separated into three regions. The first region consists of noncoding upstream regulatory region which is known as the long control region. The

second region is also called early region which contain the proteins that are involved in viral replication such as ORFs, E1, E2, E4, E5, E6, and E7. The third region contains L1 and L2 proteins which encodes viral capsid proteins and form the the protein shell they are also known as late region as show in figure 2.8.

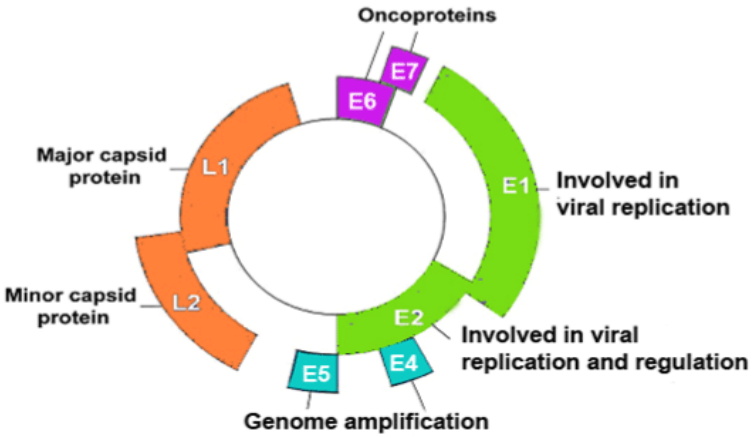


Figure 2.8 The genomic mapping of HPV-16 genes.

Source: Kajitani et al., 2012

2.2.2 Pathogenesis of Cervical Cancer

All genes of HPV contribute differently for its pathogenesis. E1 plays an important role is the transformation of HPV because in the absence of E1 HPV is not able to replicate in host cell. E2 interacts with promoters to regulate the process of viral replication and transcription in host cell. E5 bind with growth factors to down regulate the major histocompatibility complex class I molecules. Two main oncogenes that are involved in

host cell proliferation are E6 and E7 because of their continuous expression throughout their life cycle. The exact role of E4 in pathogenesis is unidentified, but only thing known is that as E4 is part of early region but expressed late in the life cycle. L1 and L2 encodes the major and minor portions of the capsid respectively. L1 is known for self-assembling into virus-like particles. Thus the virus is encapsidated and leaves the infected cells as an infectious agent which is ready to further infect the normal basal cells of squamous epithelium (Stanley, 2012). Once inside the cell the DNA is present at a low level as an episome, and the viral genome can replicate as the host cell matures. After the genes are transcribed, they exit the nucleus and undergo processing. In the case of the capsid proteins later in the life cycle, they re-enter the nucleus for assembly after which they exit the cell completely.

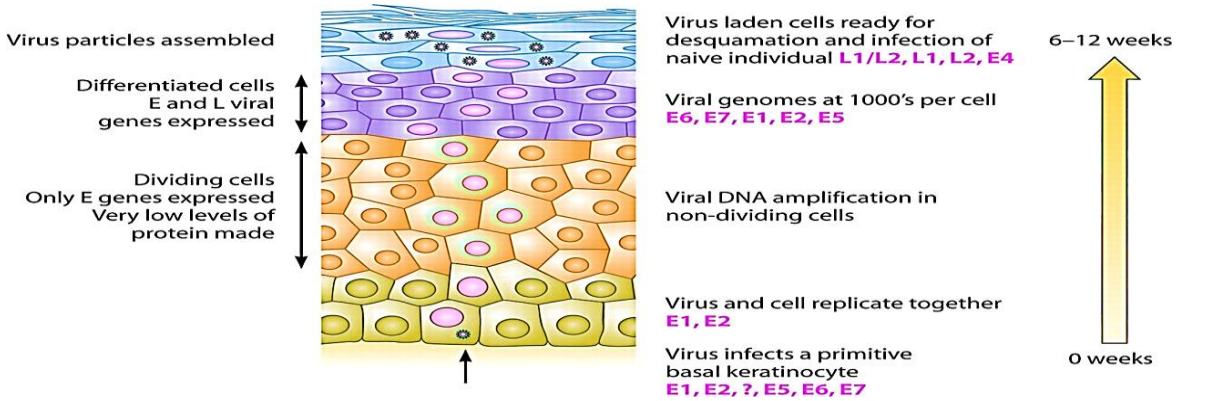


Figure 2.9: Infectious lifecycle of HPV

It takes about 3 weeks to form an infectious viral particle. It has a very long cycle and has no blood born phase thus doesn't cause cell death. Due to which it's an excellent and successful pathogen as shown in figure 2.9. These genes facilitate the entry into the host cell and can differ from type to type

2.2.3 HPV in Cervical Cancer

For the first time in 1949 electron microscope was used for detecting human papillomavirus and afterwards in 1963 HPV DNA was identified, but the role of HPV in cervical cancer was not identified yet. In 1980, the association of HPV in cervical cancer was first reported by Harold zur Hausen. From these findings it was concluded that HPV is associated in almost all cervical cancers with the implication of High risk types. It is generally accepted that HPV cause cervical cancer in women which is the the most common cancer in women worldwide. In 1996, the World Health Association and European Research Organization on Genital Infection and Neoplasia and the National Institutes of Health Consensus Conference on Cervical Cancer, accepted HPV as main reason for cervical cancer.

In the past group of studies and international review parties strongly correlates that Human papillomavirus shows strong association with cervical cancer. In another study it has been shown that out of these 40 HPV types found in the genital tract, HPV-16 accounts for most of the cervical cancer cases followed by HPV-18, HPV 45, and then

HPV 31 globally, they also concluded that HPV-16 accounts more for squamous cell carcinoma whereas HPV-18 accounts more for adenocarcinoma but the basis of these findings are unknown. Another study with same results were performed in 2005 which shows that the chances of getting affected with this cancer is considerably higher in women exposed to HPV-16 or 18 than other HPV types.

In the past different studies showed strong association of human papillomavirus with cervical cancer in different countries and concluded that HPV was detected in 93% of cancers with majority of cases positive for HPV-16. These studies also conclude that HPV-16 predominated in squamous cell carcinoma than adenocarcinoma which is more prevalent in HPV-18 (Bosch et al., 1995).

In 2004 research done to check the distribution of different types of HPV in cervical cancer worldwide and they found that HPV-16 and 18 are constantly involved in cancer of all region as shown in Figure 2.10.

Human papillomavirus is responsible for about 5% of all cancers globally and hence conflicting data available which shows the involvement of HPV in different cancers.

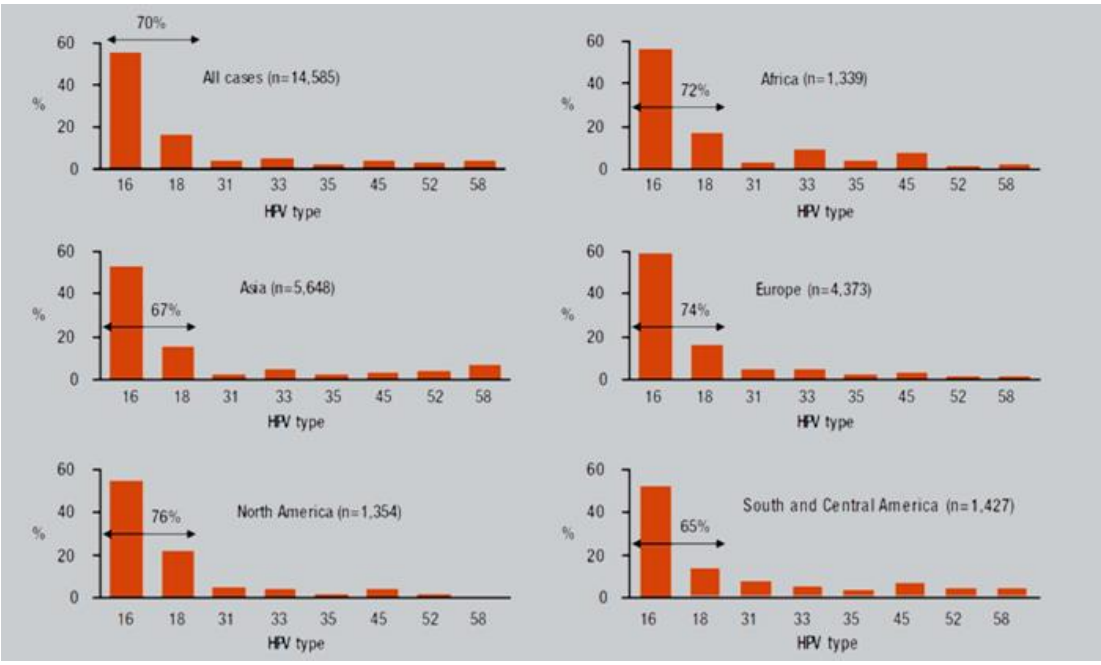


Figure 2.10 Percentage of Cervical Cancer attributes to most prevalent high risk HPV genotypes by region

Source : (Arbyn et al., 2011)

2.3 HPV in Anogenital Cancers

Out of genital types of HPV high risk HPV-16 and 18 shows strong relation in the development of anogenital cancers. The studies on the notorious role of HPV in other genital cancers is inadequate but the obvious epidemiological studies indicate that cancers of the vagina and anus caused by HPV. HPV is involved in 90% of vaginal cancers and 85% of anal cancers. Once again studies concluded that among all HPV positive anogenital cancers HPV-16 is the the most prominent.

2.3.1 HPV in Prostate cancer

Prostate cancer is the development of cancer of prostate gland in the reproductive system of male which makes fluid that forms part of semen. The prostate lies just below the bladder and in front of the rectum (Taplin et al., 1995) as shown in fig 2.11.

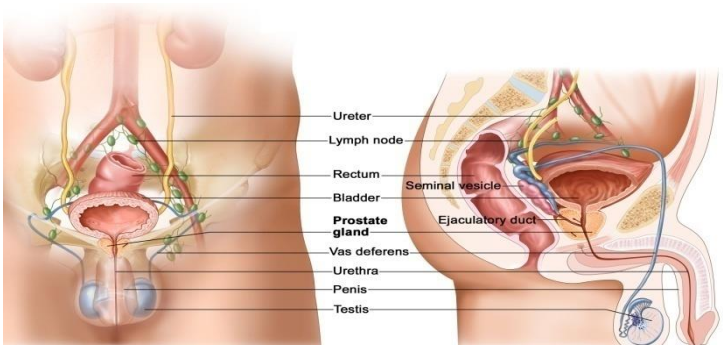


Figure 2.11Anatomy of Male reproductive and urinary system showing prostate and other organs

Source: Taplin et al., 1995

The role of human papillomavirus in prostate cancer is highly notorious a lot of studies are carried out to investigate the role of HPV in prostate cancer but concluded with contradictory results, because some studies suggest a positive association between HPV infection and prostate cancer, while other studies do not show any positive correlation between HPV and prostate cancer (Korodi et al., 2005). In 2010 Martinez-Fierro and his coworkers confirm the presence of HPV in large sample of prostate cancer which shows significant association between HPV and prostate cancer (Martinez-Fierro et al., 2010)

In the same year other group of studies by using different techniques concluded negative association in large number of samples. (Aghakhani et al., 2011; Groom et al., 2012; Hrbacek et al., 2011; Rogler et al., 2011). Again in 2010 a different group of study investigated the type specific HPV in prostate cancer but unable to find positive association of HPV-16 and 18 in prostate cancer (Sutcliffe et al., 2010). More recently a number of studies carried out to find distribution of type specific HPV in prostate cancer and find positive prevalence of large number of HPV-16 in prostate cancer (Leiros et al., 2005; Lin et al., 2011). In 2013 Whitaker et al confirm the role of HPV-18 in prostate cancer (Whitaker et al., 2013) on the other hand in another study no involvement of HPV-16 and 18 rather concluded the role of HPV 33 in prostate cancer.(Adami et al., 2003) Therefore, these mixed results are consistent with the present controversial landscape on the role of HPV infection in prostate carcinogenesis. To find out the solution of this dispute about the role of HPV in prostate cancer their needs additional research to give the answer.

2.3.2 HPV in Endometroid cancer

Endometroid cancer is the cancer of endometrium (Amant et al., 2005) shown in figure 2.12. The aetiologic role of human papillomavirus (HPV) in a variety of malignancies of cervix is well established now the presence of HPV in endometrium has also been reported, although less commonly.

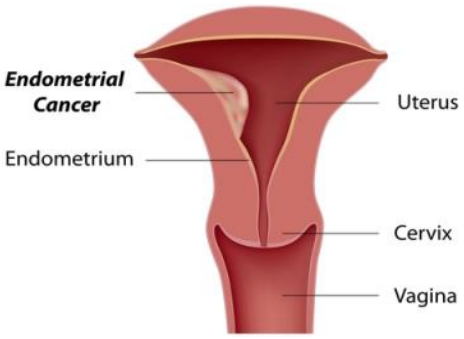


Figure 2.12 Showing Endometroid Cancer

Source: (Amant et al., 2005)

In a group of study Fujita and his coworkers investigated the role of HPV in endometroid carcinoma by using different techniques such as PCR, in situ hybridization and southern blotting in a large group of sample from different regions, they find out positive association of HPV in endometroid cancer. Numbers of positive samples vary in different techniques. Their study suggest that HPV, especially HPV-16, may play an aetiologic role in endometrioid adenocarcinoma (Fujita et al., 1995). In 1998 another study concluded that HPV type 16 was found to be involved in majority of samples (Lininger et al., 1998). Lai et al and his colleagues in 1992 analyzed both ovarian and endometrial cancer samples using PCR. HPV were detected in both benign and malignant tumor samples but in this study as contrary to other studies HPV-18 was found to be involved more as compared to HPV-16 (Lai et al., 1992). In 1994 another study was proposed by same group of people, in this study they used reverse transcription PCR to check the presence of HPV-16 and 18 in both ovarian and endometrial carcinomas. Their study confirm that HPV-16 were detected in more proportion as compared to HPV-18 (Lai et al., 1994). On the other side a number of studies also carried out which shows negative association of HPV in endometroid cancer but still their remains a controversial issue that how HPV causes malignancy (Brewster et al., 1999). By these studies we can predict that HPV is not related to prognostic parameters and survival and thus little information with regard to the viral presence in endometrial hyperplasia (Semczuk et al., 2000).

2.3.3 HPV in Ovarian cancer

Ovarian cancer is a cancer that begins in an ovary. It results in abnormal cells that have the ability to invade or spread to other parts of the body. Ovarian tumor is a common neoplasm of the female ovaries is the most frequent cause of death from gynecological malignancies (Platz & Benda, 1995) shown in figure 2.13.

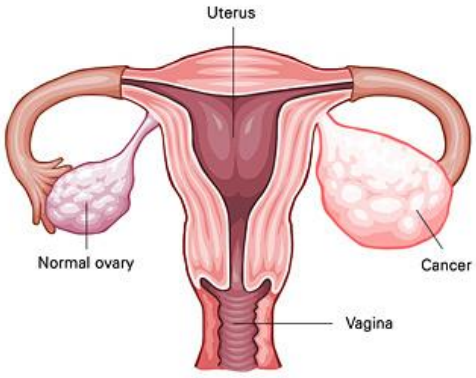


Figure 2.13: ovarian cancer

Source: (Biswas & Dey, 2011)

In 1987 for the first time ovarian tumors analyzed for HPV in by Kaufman and his fellows and they analyzed a series of patients with advanced ovarian adenocarcinomas and shows positive association in almost all cases but these results were retracted a year afterward by the authors, stating that they were in error (Kaufman et al., 1987). Lai et al and his colleagues in 1992 analyzed both ovarian and endometrial cancer samples using PCR. HPV were detected in both benign and malignant tumor samples but in this study as contrary to other studies HPV-18 was found to be involved more as compared to HPV-16 (Lai et al., 1992). Reverse transcription PCR to check the presence of HPV-16 and 18 in

both ovarian and endometrial carcinomas. Their study confirm that HPV-16 were detected in more proportion as compared to HPV-18 (Lai et al., 1994).

A lot of studies investigated the role of HPV in ovarian cancer but some studies unable to find positive association (Anttila et al., 1999; Beckmann et al., 1991; Leake et al., 1989; Runnebaum et al., 1995). We found a high prevalence of HPV-positive DNA in ovarian cancer cases, but the role of HPV in ovarian cancer remains inconclusive. Further studies are needed to found the main role.

2.3.4 HPV in Rectal Cancer

Rectal cancer is the development of cancer in the rectum. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body. Signs and symptoms may include blood in the stool, a change in bowel movements, weight loss, and feeling tired all the time (Zhang et al., 2011).

Rectal cancer is the growth of abnormal cancerous cells in the lower part of the colon that connects the anus to the large bowel. Actual cause of Rectal cancer is not known, but risk factors include increasing age, smoking, family history, high-fat diet, or a history of polyps but now in recent years controversial role of HPV in rectal cancer is increasing but defined results are still undergoing. For this purpose a lot of research is conducting in order to determine if a strong relationship exists between HPV infection and rectal cancer. In earlier times group of studies shows the prevalence of HPV in rectal cancer

significantly. Among these cases, HPV frequency decreases with increasing severity of the disease yet, it remains to be seen whether the association of HPV with the earlier stages of colon adenocarcinoma is a reflection of a true absence of viral infection in the advanced stages or simply a biologic consequence of viral integration (Perez et al., 2005). A lot of studies are in consistent with this study (Cheng et al., 1995; McGregor et al., 1993; Salepci et al., 1999). In 1992 further studies carried out to find the role of HPV in colon cancer using PCR but they are unable to find positive association between HPV and colon cancer (Shah et al., 1992; Shroyer et al., 1992).

In 1990 Kirgan and his coworkers found the same distribution of the virus throughout the colon, mere presence of HPV in human tumours supports a possible association with colorectal cancer but definitively does not confirm the aetiological role (Kirgan et al., 1990).

In most recent study a large sample of colorectal carcinomas checked for prevalence of HPV but they are found to be very low. Prior studies detecting high HPV prevalence in colorectal carcinomas are likely the result of contamination from the anal canal or clinical processing (Burnett et al., 2013). HPV has been detected in the majority of reported series, but published literature lacks indefinite data regarding standard method of investigation and stratification of groups and population. These data encourage further studies with the aim to investigate the presence of the virus in larger series, its possible role in oncogenesis, the integration in host genome, the expression of viral oncoproteins, the mutations in HPV positive cancers and routes of colon infections.

In order to find definitive data on the role of HPV in colon cancer there is emergent need of further evaluation however, it is clear that this is a promising area and the available data still do not permit to confirm conclusions.

2.3.5 HPV in Vaginal Cancer

Vaginal cancer is any type of cancer that forms in the tissues of the vagina. Primary vaginal cancer is rare in the general population of women and is usually a squamous-cell carcinoma. Metastases are more common. Vaginal cancer occurs more often in women over age 50, but can occur at any age, even in infancy. It often can be cured if found and treated in early stages. Surgery alone or surgery combined with pelvic radiation is typically used to treat vaginal cancer (Stock et al., 1995).

Human papillomavirus (HPV) is a common infection and is passed from one person to another by sexual contact. Around 8 out of 10 people (80%) in the UK are infected with the HPV virus at some time during their lifetime. For most people the virus causes no harm and goes away without treatment. It is only when the infection won't clear up that sometimes there is a problem. But most women infected with HPV don’t go on to develop vaginal cancer.

HPV is present in two thirds of women who have vaginal cancer (66%). There are many different types of human papillomavirus (HPV). HPV types 6 and 11 can infect the female and male genital organs and the anal area, causing visible genital warts. Women

with these types of the virus have an increased risk of developing precancerous cell changes and some may develop vaginal cancer (Daling et al., 2002).

Women with HPV types 16, 18 and 31, as well as some others have a higher risk of developing genital and anal cancers. But the type of HPV most strongly linked to vaginal cancer is HPV-16. This type of HPV can cause changes in the cells covering the vagina. The changes make the cells more likely to become cancerous in time. But this can take years. Most women infected with this virus do not develop cancer of the vagina some other factors must also be needed.

2.3.6 HPV in Lungs Cancer

Lung cancer is the uncontrolled growth of abnormal cells that start off in one or both lungs. The abnormal cells do not always develop into healthy lung tissues, sometimes they divide rapidly and form tumors (Prajapati, 2014) shown in figure 2.14.

Figure 2.14: Lungs cancer

Source: (Prajapati, 2014).

Lungs cancer is leading cause of death worldwide both in males and females. In addition to smoking other environmental factors also responsible for lungs cancer. Main aetiological factor responsible for lungs cancer is controversial. Human papillomavirus (HPV), known to be an aetiological agent for genital cancers, has been suggested also to be a possible contributory agent for lungs

cancer (Prabhu et al., 2012). Different studies performed to investigate the role of HPV in lungs cancer and concluded that HPV is second leading cause of lungs cancer after smoking. For the first time in 1974 a study conducted which confirm the role of HPV in bronchial lesions (Roglic et al., 1974) after sometimes in 1978 another study confirm the the involvement of HPV in lugs cancer (Rubel & Reynolds, 1978). When significant association of lungs cancer and HPV were reported for the first time then in 1979 a study was conducted to analyze the changes in cancer patient and concluded that epithelial changes that arises in lungs cancer patient closely resemble HPV induced genital lesions (Syrjanen, 1979). A number of studies conducted which shows negative association of HPV in lungs cancer (Gorgoulis et al., 1999; Iwakawa et al., 2010; Koshiol et al., 2011; Lim et al., 2009).

After investigating the association a lot of research employed to determined type specific HPV in lungs cancer and concluded with the result that HPV-16 is found to be more prevalent in cancer patients followed by HPV-18 and HPV-6 (Baba et al., 2010; Castillo et al., 2006; Syrjanen et al., 1989). In addition to all these studies their still needs to find main role of HPV in lugs cancer.

2.3.7 HPV in Breast Cancer

Breast cancer is a malignant tumor that starts in the cells of the breast. A malignant tumor is a group of cancer cells that can grow into surrounding tissues or spread to distant areas of the body. The disease occurs almost entirely in women, but men can get it, too.

High risk HPV has been recognized as being causal in almost all cervical cancers. In recent years, evidence indicates that HPV may also have a role in breast cancer. The involvement of the virus in breast cancer remains questionable. Earlier reports have shown that HPV DNA was detected in breast cancer specimens and the prevalence of HPV positive breast cancer vary from one study to another (Lawson et al., 2006). In 2004 a group of study conducted which includes breast cancer patients and patients with both cervical cancer and breast cancer, after investigation association of HPV is positive in patients with both carcinomas and among them HPV-16 is involved in majority of cases while no HPV detected in patients with breast cancer alone (Widschwendter et al., 2004). High risk types 16, 18 and 33 of HPV have been screened in breast cancers patients from a wide group of different populations using different techniques. This study shows that oncogenic characteristics of HPV associated breast cancer related to HPV-associated cervical cancer (Lawson et al., 2006).

After this study another study came out in 2009 which shows that the oncogenic properties of these HPV-associated breast cancers were comparable to HPV-associated cervical cancer and hence predicted that the pathogenesis of cervical cancer caused by HPV may share a similar mechanism to cause breast cancer (Heng et al., 2009). This theory has been further exemplified with the discovery of koilocytosis in breast cancer cells because it is well documented in HPV-associated cervical cancer cells and hence provides the evidence that HPV may play a causal role instead of being merely a passenger in the pathogenesis of the disease (Lawson et al., 2009)

In a recent study on breast cancer cell lines, high risk HPV type 18 have been seen in the nuclei of breast tumor cells (Li et al., 2011).In addition to analyze the presence of HPV-16 or 18 in breast cancer samples another study demonstrate the role of HPV33 in breast cancer samples. A lot of research done on finding HPV in breast cancer but still needs to find aetiological role of HPV in Breast cancer because additional lines of evidence need to be obtained in order to assess the possibility of breast cancer prevention using HPV vaccines.

2.4 Vaccine Development

The alarming number of cases of cervical cancer and other anogenital cancers requires effective treatment and preventive vaccine so as to eradicate the cancer from the community. In the past two commercially available prophylactic vaccines designed against HPV infection called as Gardasil and Cervarix. Gardasil is a quadrivalent vaccine directed against low-risk HPV types 6 and 11 and high-risk HPV types 16 and 18 (Waknine, 2006). Cervarix is a bivalent vaccine which is directed against high-risk HPV types 16 and 18 (CDC, 2010).

A major breakthrough in HPV vaccine technology was the demonstration that L1 has the intrinsic capacity to self-assemble VLPs. These are morphologically indistinguishable from infectious virions although they lack viral DNA. They present conformational epitopes that are highly immunogenic. The co-expression of L2 can increase the in vitro production of VLPs, but there is no evidence that it increases immunogenicity.

These HPV vaccines targeting HPV16 and HPV18 have the potential to produce a dramatic drop in cervical cancer rates if they are widely used. Nevertheless, the licensed vaccines are currently too costly for sustained global implementation and thus to reach those populations that would benefit most from such a vaccine(Gupta et al., 2013) . It is important to note that over 80% of cervical cancer cases occur in developing nations.

This reflects the lack of cytological screening programs and intervention in these countries. In these settings, therefore, the cost of HPV vaccination must be borne in addition to that of screening. Thus, it would be highly desirable to develop a more affordable vaccine for the developing world. Vaccine and cancer prevention strategies for cervical cancer depend on the medical/economic situations of each country.

In low-resource settings, subunit based peptide vaccines against HPV infections have clearly the potential to reduce incidence of cervical cancer cost effectively. The current HPV vaccines are known to have a marked ability to induce neutralizing antibodies but because of limitations of the current HPV vaccines as mentioned above, necessity of the subunit peptide based vaccine program are implemented in the world.

2.4.1 Molecular characterization of HPV-16 L1

Diverse decrease in rate of cervical cancer due to the use of HPV prophylactic vaccine but still there is a need of vaccine which is subunit vaccines based on the ability of the viral L1 major capsid protein to form virus-like particles (VLPs) that meet the demands

of current situation. Currently there is not enough data on local isolates of HPV-16 so to detect high risk HPV in cervical cancer we characterize L1 gene by using in-silico analysis, via PCR based gene amplification and sequencing which will help in designing prophylactic vaccine that is utmost necessity of current time in our country.

To meet the demands novel vaccines should be designed on the basis of molecular technology for eliciting proper immune response against HPV, including both broadly neutralizing antibodies and effective T-cell response. The concept of vaccines is based on identification and chemical synthesis of B-cell and T-cell epitopes which are immunodominant and can induce specific immune responses. The accelerating growth of bioinformatics techniques and applications along with the substantial amount of experimental data makes it easy to design a sububit vaccine against HPV infection with minimal resources.

B-cell epitope of a target molecule can be conjugated with a T-cell epitope to make it immunogenic. Epitope-based vaccines can be constructed for T and B lymphocytes. The T-cell epitopes are typically peptide fragments, whereas the B-cell epitopes can be proteins, lipids, nucleic acids or carbohydrates. Peptides have become desirable vaccine candidates owing to their comparatively easy production and construction, chemical stability, and absence of infectious potential. The epitope is recognizable by the immune system part of the antigen, and in particular by antibodies, B cells or T cells. The epitopes may belong to both foreign and self proteins, and they can be categorized as conformational or linear, depending on their structure and integration with the paratope.

T-cell epitopes are presented on the surface of an antigen-presenting cell (APC), where they are bound to major histocompatibility (MHC) molecules in order to induce immune response.

Immunogenic epitopes used as peptide vaccines is promising approach for HPV. However, appropriate design and primary in-silico analysis is an essential prerequisite before commencing costly transgenic animal studies. Accurate prediction of peptide immunogenicity and characterization of relation between peptide sequences and peptide immunogenicity will be greatly helpful for vaccine designs and understanding of the immune system.

2.5 Objectives

Main objectives of this study are as follows:

1. Retrospective screening of various cancer biopsies, for the presence of HPV DNA followed by subtype screening of HPV-16 and 18
2. Phylogenetic Analysis of Pakistani variant of HPV16- L1 with L1 of other HPV genotypes using Bioinformatics tools
3. Sequence analysis of HPV16- L1 from our local variants.
4. To predict conserved B-cell and T-cell epitopes in HPV-16-L1 of Pakistani variant cloned from infected patients of all HPV genotypes around the globe by using online bioinformatics tools.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

The tissue samples in formalin fixed paraffin embedded (FFPE) form patients suffering from different cancers were collected from pathology labs of different hospitals i.e. PIMS Islamabad, Fauji Foundation hospital Rawalpindi, AFIP Rawalpindi during 2011 to 2015, along with the patient history about age, sex and lymph node status. The hospital provided us with FFPE tissue sections 25 µm in thickness.

3.2 DNA extraction

DNA extraction was carried out from FFPE tissue biopsies using protocol described here.

Genomic DNA Extraction from tissue biopsies

20-30 µm thick section of sample cut from tissue biopsy sample on microtome. These sections were placed in 1.5ml eppendorf tubes.

3.2.1 Method

The method of extraction of DNA from tissue biopsy is as follows

- Deparaffinization

Tissue section was deparaffinized with 500 µl of 100% xylene. The contents were vortexed for 1 minute and then left in water bath for 15 minutes at 65ºC. The xylene was removed from tube, repeat the procedure twice so that all paraffin removed from tissue.

- Xylene removal

  To remove xylene residues, the sample was washed five times with Ethanol as follows.

  1ml of absolute ethanol was added in tube, vortexed for ten seconds and then removed after 10 minutes. 1ml of absolute ethanol was added and vortexed for ten seconds and then removed after 30 minutes. 90% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and then ethanol was removed. 1ml of 70% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and ethanol was removed. 1ml of 50% ethanol was added and mixed by vortex for ten seconds and after 20 minutes, tube was centrifuged at 8000 RPM and ethanol was removed. The microtube was then left in a 40ºC oven in order to dry the tissue.

- Digestion of Proteins

  After the tissue was dried, 500ul of tail buffer was added and then sample tube was incubated at 55ºC for overnight so as to allow digestion by proteinase K. The tail buffer recipe is given in the table

- Precipitation and isolation of DNA

  The eppendrof tube containing the tissue sample was taken and was placed in the water bath at 55°C for 30 minutes. After which it was centrifuged for 5 minutes at 14000 RPM. 200µl of saturated 6M NaCl was added to the tube after it was taken out from the

centrifuge. The tube was vortexed for 1 minute and was centrifuged again for about 15 minutes at 1400 RPM. A new eppendrof tube was prepared meanwhile. After centrifuge was complete a pellet of tissue can be seen at the bottom of the tube. The supernatant was taken from the first tube and was transferred to the new eppendrof tube. The top phase or the pellet was not taken. About 300-500µl supernatant was taken in the new tube and the old tube was discarded. 500µl Isopropanol was added to the supernatant tube; it was vortexed for 2 minutes and followed by 10 minutes centrifuge. When the eppendrof tube was taken out of the centrifuge, a small pellet was visible at the bottom of the tube. The

Ingredients	Concentration	Volume
EDTA	100mM	50ul
Tris HCL	500mM	25ul
NaCl	100mM	50 ul
Proteinase K	20mg/ml	12.5ul
SDS	10%	50ul
Distilled Water	-----	312.5ul

Table 3.1 The recipe of tail buffer

supernatant was discarded and the pellet was dried. The pellet was washed with 1ml 70% ethanol and was centrifuged again for 10 minutes The supernatant was discarded and the pellet was dried which was later re- suspended in 100- 200-µl TE depending on the size of the pellet. The tube was incubated for 2 hours at 37°C with gentle shaking. This

freshly extracted DNA could now be used for gel electrophoresis, PCR or can be stored at 4°C.

3.3 Analysis of DNA through Agarose Gel Electrophoresis

To analyze the integrity of genomic DNA isolated from tissue biopsies, 0.8 % agarose gel was prepared in 1X Tris Acetate EDTA (TAE) and was run in the same buffer composition. 10X TAE buffer (pH 8.3) was prepared as stock solution by dissolving 7.5 g EDTA, 55 g of glacial acetic acid and 108 g Tris base in 800 mL of distilled water and volume was adjusted to 1 liter by adding distilled water. For gel preparation, working solution of TAE was prepared by diluting the stock solution by 1:10 with distilled water. For making gel, 0.8g agarose was mixed in 100mL of 1X TAE and to dissolve agarose the mixture was heated in microwave oven. The gel mixture was cooled to ~60 °C and upon cooling, 5 µL of ethidium bromide (10 mg/mL) was added to stain the gel. To analyze, 2 µL of DNA from each sample was run on the gel along with 6X loading dye. This gel was run at a 120 volts for 15 minutes. Gel was visualized under ultr-violet light and photographed by gel documentation system (Wealtec, Sparks, USA).

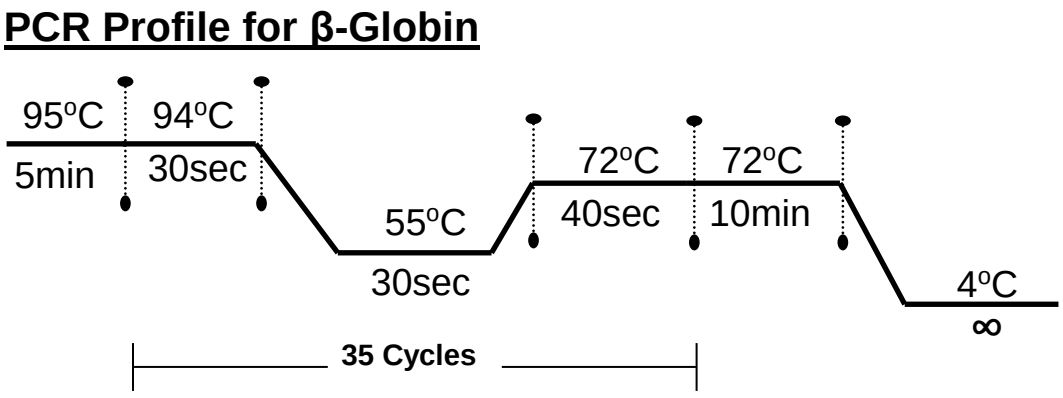
3.4 Polymerase Chain Reaction

Firstly, Polymerase chain reaction (PCR) was carried out to check the integrity of DNA for human β-globin gene as used by Daniel et al., (2011). When integrity of samples were checked, general primers (GP) were used to screened the samples for presence of HPV.

For the identification of HPV genotype 16 and 18 specific primers Ts16 and Ts18 were used which targeted at the E-6 and L1 genes respectively.

3.4.1 Optimization of PCR Conditions

The DNA integrity was confirmed using PCR for human β-globin gene as used by Daniel et al., (2011). The 12.5 µL PCR reaction mixture consisted of Taq polymerase buffer (1x), MgCl2 (2mM), 2 mM of dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20pmole each), thermostable Taq polymerase (1U) and 20 ng of DNA as template. The nuclease free water was added to make the volume up to 12.5 µl. PCR reaction was carried out in thermocycler and following PCR profile was used.

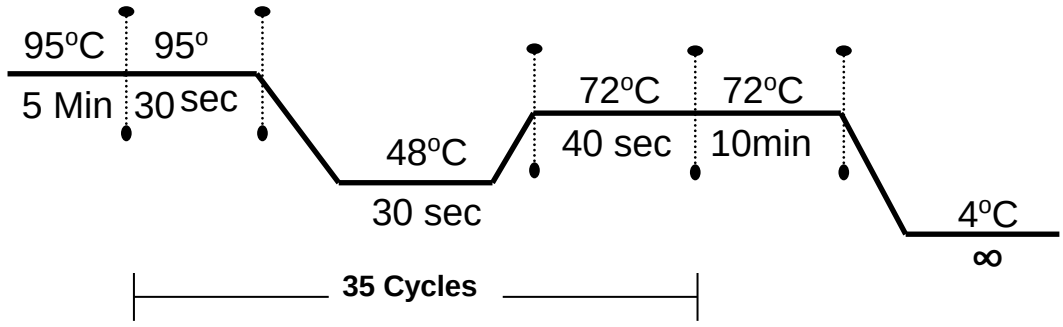


To analyze the PCR products 2% agarose gel was prepared in 1X TAE and 8 µL of PCR product was run on the gel along with 6X loading dye. This gel was run at a constant current of 120 volts for 20 minutes. Gel was visualized under ultra-violet light.

3.4.2 Detection of HPV using General Primers (GP)

The general primers for HPV were used for its identification which targeted the conserved region in the L1 gene of all the HPV strains in general. Extracted DNA was subjected to PCR amplification, using primers of GP5/GP6. For amplification of gene, the 12.5 µL PCR reaction mixture consisted of Taq polymerase buffer (10X), MgCl2 (2mM), 2mM dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20pmole each), thermostable Taq polymerase (1 U) and 20 ng of DNA as template. The nuclease free water was added to make the volume up to 12.5 µL.PCR mixture was placed in thermo cycler and run on following profile.

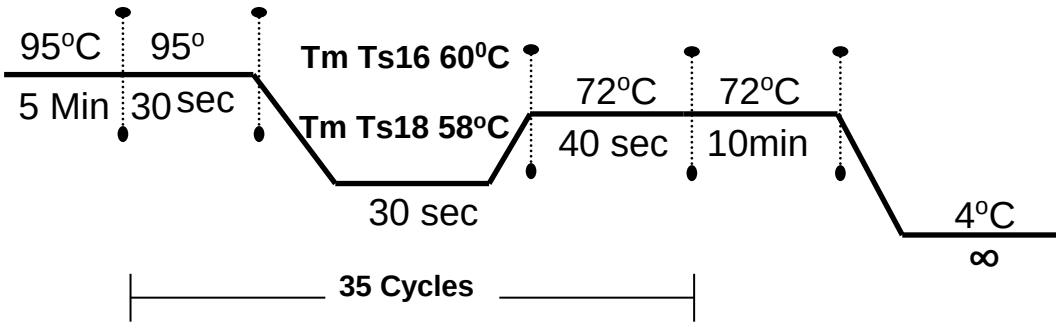
PCR Profile for GP



To analyze the PCR products 2% agarose gel was prepared in 1X TAE and 8 µL of PCR product was run on the gel along with 6X loading dye. This gel was run at a constant current of 120 volts for 20 minutes. Gel was visualized under ultra-violet light.

3.4.3 Detection of HPV genotypes HPV-16 and 18

To detect the type specific HPV genotype DNA was subjected to PCR amplification using detection primers Ts16 and Ts18, the 12.5 µL PCR reaction mixture consisted of Taq polymerase buffer (10X), MgCl2 (2mM), 2 mM dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20pmole each), thermostable Taq polymerase (1 U) and 20 ng of DNA as template. The nuclease free water was added to make the volume up to 12.5 µL.PCR mixture was placed in thermo cycler and run on following profile.

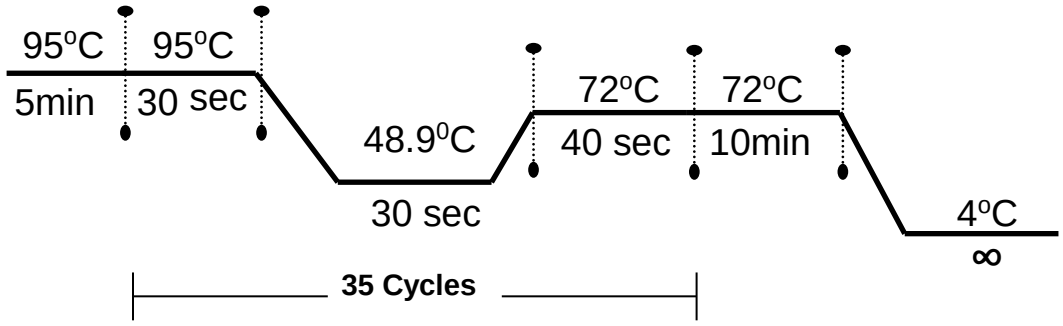


To analyze the PCR products 2% agarose gel was prepared in 1X TAE and 8 µL of PCR product was run on the gel along with 6X loading dye. This gel was run at a constant current of 120 volts for 20 minutes. Gel was visualized under ultra-violet light.

3.4.4 PCR Amplification of HPV-16 L1

Extracted DNA was subjected to PCR amplification, using primers designed for detection of HPV-16 L1 gene. For amplification of gene, the 12.5 µL PCR reaction mixture consisted of Taq polymerase buffer (10X), MgCl2 (2mM), 2 mM dNTPs (dATP, dGTP, dTTP, dCTP), forward and reverser primers (20pmole each), thermostable Taq polymerase (1 U) and 20 ng of DNA as template. The nuclease free water was added to make the volume up to 12.5 µL.PCR mixture was placed in thermo cycler and run on following profile.

PCR Profile for HPV-16-L1



To analyze the PCR products 2% agarose gel was prepared in 1X TAE and 8 µL of PCR product was run on the gel along with 6X loading dye. This gel was run at a constant current of 120 volts for 20 minutes. Gel was visualized under ultra-violet light.

Primer	Sequence (5'---3')	Size/bp	TM/°C	Target site	Reference
β-globin–F	ACACAACTGTGTTCACT AGC	121	5.0	β-globin	(Gillison et al., 2000)
β-globin–R	CAACTTCATCCACGT TCACC	121	55.0	β-globin	(Gillison et al., 2000)
HPV-GP-F	TTTGTTACTGTGGTA GATAC	150	48.0	L1	(Jacobs et al., 1997)
HPV-GP-R	GAAAAATAAACTGT AAATCA	150	48.0	L1	(Jacobs et al., 1997)
HPV-TS16-F	GGTCGGTGGACCGG TCGATG	96	60.0	E6	(Baay et al., 1996)
HPV-TS16-R	GCAATGTAGGTGTAT CTCCA	96	60.0	E6	(Baay et al., 1996)
HPV-TS18-F	CCTTGGACGTAAATT TTTGG	115	58.0	L1	(Baay et al., 1996)
HPV-TS18-R	CACGCACACGCTTG GCAGGT	115	58.0	L1	(Baay et al., 1996)
HPV16-L1-F	ATGTCTCTTTGGCTG CCTAG	1500	60.4	L1	Currently designed
HPV16-L1-R	TTACAGCTTACGTTT TTTGCGTTTA	1500	58.0	L1	Currently designed
HPV16-L1-F Partial sequence	GTTGTTGATACTACA CGCAGTA	456	48.9	L1	Currently designed
HPV16-L1-R Partial sequence	TGCATAAGCACTAG CATTTTCTG	456	48.9	L1	Currently designed

Table 3.2: Details of all Primers

3.4.5 Elution

The GeneJET™ PCR Purification Kit is designed for rapid and efficient purification of DNA from PCR and other enzymatic reaction mixtures. The GeneJET PCR Purification Kit effectively removes primers, dNTPs, unincorporated labelled nucleotides, enzymes and salts from PCR and other reaction mixtures. The kit can be used for purification of DNA fragments from 25 bp to 20 kb. The recovery rates are 90-100% in a 100 bp – 10 kb DNA fragment size range (Boom et al., 1990).

- Add a 1:1 volume of Binding Buffer to PCR mixture , mix thoroughly.
- Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
- If the DNA fragment is larger than 500 bp, add a 1:2 volume of 100% isopropanol e.g., 100 µL of isopropanol should be added to 100 µl of PCR mixture combined with 100 µL of Binding Buffer. Mix thoroughly. If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
- Transfer up to 800 µL of the solution from step 1 to the purification column. Centrifuge for 30-60 s. Discard the flow-through.

- Add 700 µL of Wash Buffer diluted with the ethanol to the purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
- Centrifuge the empty purification column for an additional 1 minute to completely remove any residual wash buffer.
- Transfer the purification column to a clean 1.5 mL microcentrifuge tube.
- Add 50 µL of Elution Buffer to the center of the purification column membrane and centrifuge for 1 min.
- Discard the purification column and store the purified DNA at -20°C.

3.5 Sequencing

The eluted and purified DNA samples of L1 gene in the previous section were sent to Eurofins, Europe along with 90ul of forward primer HPV16-L1-F and Reverse primer HPV16-L1-R each. These sequences can later be used for mutational analysis of HPV-16-L1 gene in Pakistan.

3.6 Molecular characterization of HPV-16 L1

The phylogenetic tree of HPV16 L1 and L1 of other HPV genotypes were constructed using CLC workbench, 6.4. Neighbor joining algorithm and UPGMA methods were used. CLC workbench is a powerful bioinformatics tool for sequence alignment and phylogenetic analysis of the aligned sequences.

3.7 In silico analysis of HPV-16 L1

To analyze the sequence of HPV-16-L1 sequences of L1 of different HPV genotypes including both high risk and low risk reported worldwide were retrieved from NCBI nucleotide database. A total of 208 sequences were included in this study. All the sequences were aligned using CLC workbench and online tool multalign. These sequences were reported from different countries including Pakistan, China, Iran, India, Mexico, Canada, Solvenia, South Africa and U.S.A as shown in Table 3. 3

Serial no.	Country	HPV 6	HPV 11	HPV16	HPV18	HPV31	HPV33	HPV45	HPV52	HPV58	Total
1	China	1	2	1	2	----	----	----	13	1	19
2	Iran	----	----	31		----	----	----	----	----	31
3	Canada	----	----	32		----	8	----	----	----	40
4	Pakistan	----	----	1		----	----	----	----	----	1
5	Mexico	----	----	26		----	----	----	----	----	26
6	Solvenia	15	56	----		----	----	----	----	----	71
7	South Africa	----	----	2		----	----	----	----	----	2
8	US	----	----	----	2	7	----	5	4	4	22
	Total										208

Table 3.3 Sequence of HPV16 L1 with L1 of different genotypes

3.7.1 Online databases and bioinformatics tools

The Protein Information Resource database was used to determine molecular weight of the viral HPV-16 L1. Bioinformatics tool vaxijen was used for analyzing antigenic property of HPV-16-L1. VaxiJen predicts each of the HPV proteins for antigenicity property. VaxiJen is the server for alignment independent prediction of protective antigen. It was developed to allow antigen classification solely based on the physicochemical properties of proteins without recourse to sequence alignment

3.7.2 IEDB Immune Epitope Database

IEDB was employed to find B cell epitope and those B cell epitopes were selected after finding their conservedness among all the sequences .

3.7.3 ProPred-I and ProPred

Online immune informatics tools such as ProPred-I, and ProPred were performed to predict T-cell epitopes of HPV-16 L1 for both class I and II MHC binding by using. ProPred-I and ProPred. The ProPred-I is an online tool to predict the Class I MHC binding regions in protein antigens. ProPred server predicts Class II MHC-binding regions in an antigen sequence. The server helps to determine promiscuous binding regions that are useful in selecting vaccine candidate.

3.7.4 Epitope Conservation Analysis

From Pakistani isolate of HPV-16-L1 the predicted B-cell epitopes and T-cell epitopes were analyzed for conservation among Pakistan and all over the world. Conservation of these epitopes were not only analyzed against HPV-16-L1 but also other genotypes of HPV like HPV-18, HPV-6, HPV-11, HPV 31, HPV-33, HPV-45, HPV-51 worlwide. Those T-cell epitopes were selected that bind to maximum number of alleles. All the predicted epitopes were submitted to IEDB for conservence analysis and thus the epitopes that shows 50 to 100% conservancy were selected. Finally, all the selected conserved epitopes were analyzed for similarity with human proteome using Blast program to verify that these peptides will not trigger auto immunity

CHAPTER 4

RESULTS

4.1 HPV detection in Cervical FFPE Biopsies

The genomic DNA was extracted from positive cervical cancer and the presence of 121-bp fragment of β-globin gene confirmed the quality of DNA for subsequent PCR analysis. Among the positive cervical cancer samples only 50% of samples showed positive association with HPV using GP. Type specific primers Ts16 and Ts18 were used to identify HPV-16 and HPV-18. TS 16 targeted at the E6 coding region of HPV16 and TS 18 targeted at the L1 coding region of HPV18. When both of these primers used in combination a total of 75% samples were positive for HPV. Single infection of HPV16 detected in 64.5% of total samples and HPV-18 detected in 27.7% of total samples. 21% of total samples showed coinfection of both HPV16 and HPV-18. Their distribution shown in figure 4.1

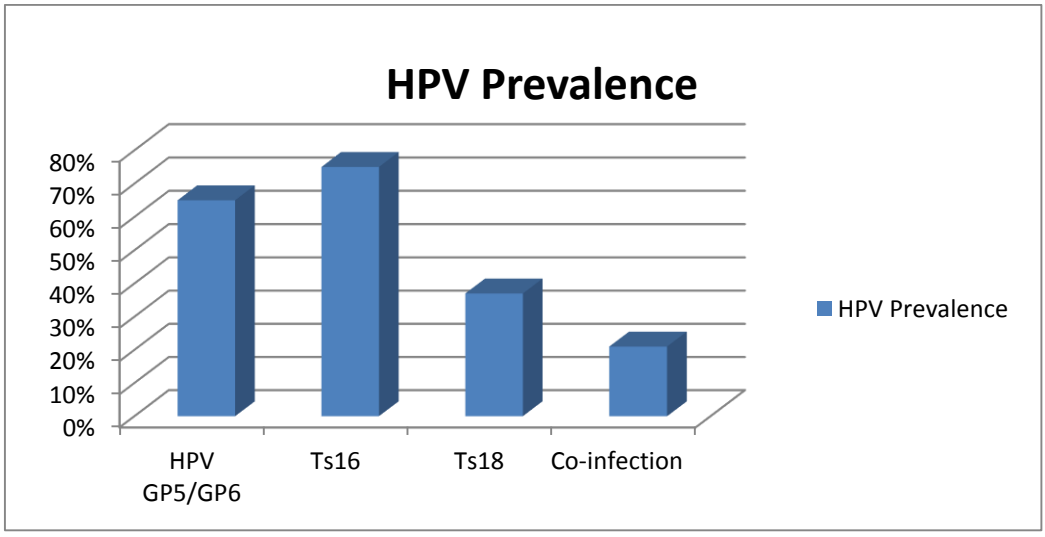
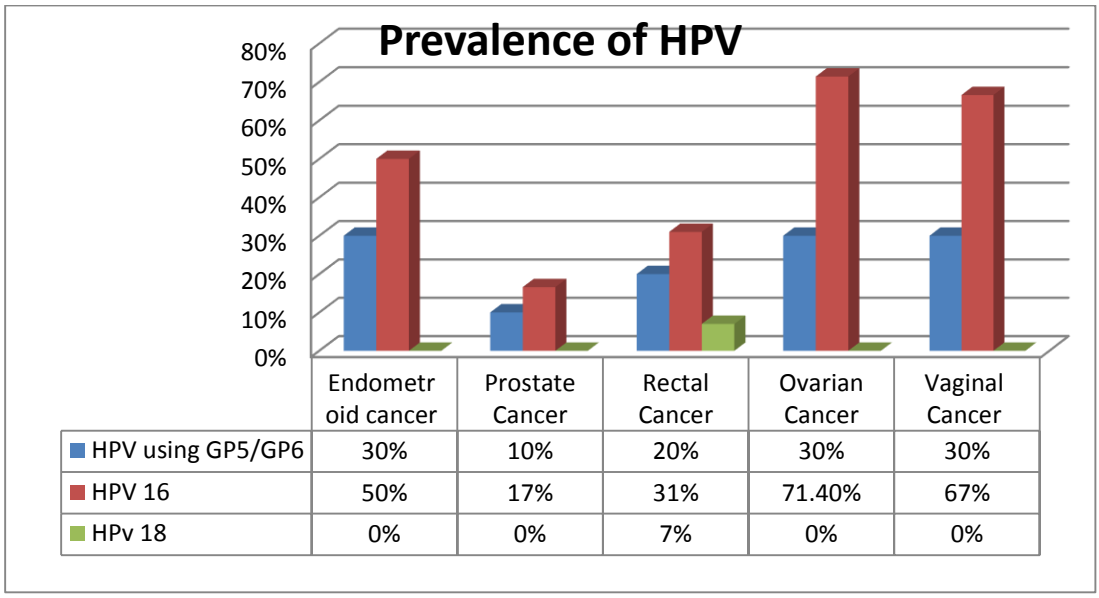


Figure 4.1 Detection of HPV prevalence using general and type specific HPV primers

Type specific primers were used because of its more sensitivity as compared to GP because our study found that some cancer samples negative for HPV by using GP can be positive when used with type specific primers that's why these samples were tested with type specific primers in order to avoid any false positive or false negative results.

4.2 Detection of HPV in Anogenital cancers

The genomic DNA was extracted from different anogenital cancers such as Endometroid Cancer, Ovarian Cancer, Vaginal Cancer, Anorectal Cancer and Prostate Cancer. The presence of 121-bp fragment of β-globin gene confirmed the quality of DNA for



	Endometroid cancer	Prostate Cancer	Rectal Cancer	Ovarian Cancer	Vaginal Cancer
HPV using GP5/GP6	30%	10%	20%	30%	30%
HPV 16	50%	17%	31%	71.40%	67%
HPv 18	0%	0%	7%	0%	0%

Figure 4.2 Prevalence of HPV in Anogenital Cancers using different sets of primers

subsequent PCR analysis. We screened these samples for HPV detection using GP and type specific primers in order to increase the accuracy of results. Results of these cancers were shown in figure 4.2

4.3 Detection of HPV in non-Anogenital cancers

The genomic DNA was extracted from non-anogenital cancers such as Breast Cancer amd Lungs Cancer. The presence of 121-bp fragment of β-globin gene confirmed the quality of DNA for subsequent PCR analysis. We screened these samples for HPV detection using GP and type specific primers. Results of these cancers were shown in figure 4.3

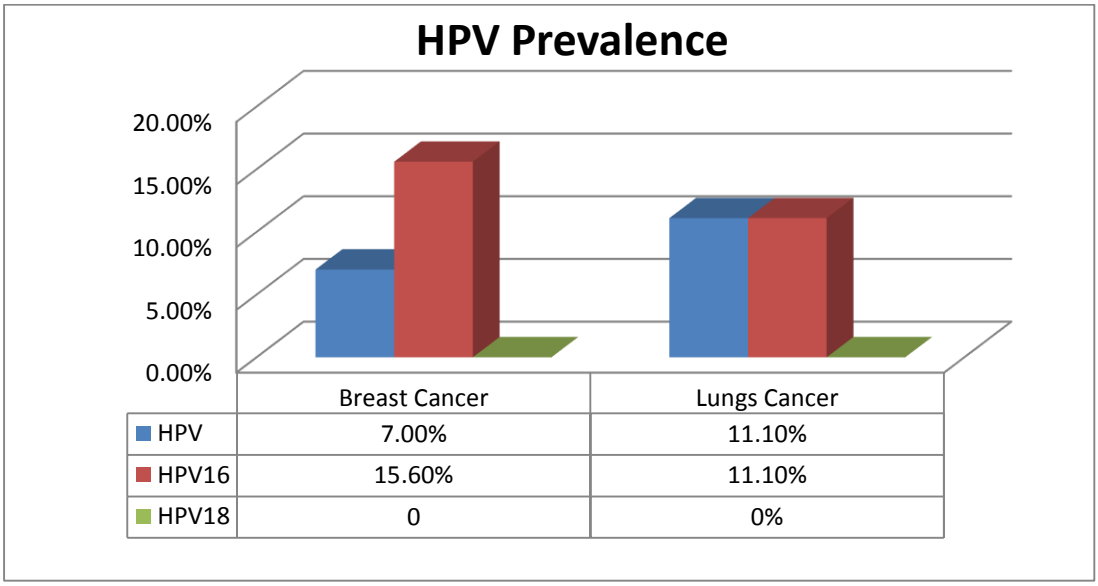


Figure 4.3 HPV prevalence in Non-Anogenital Cancers

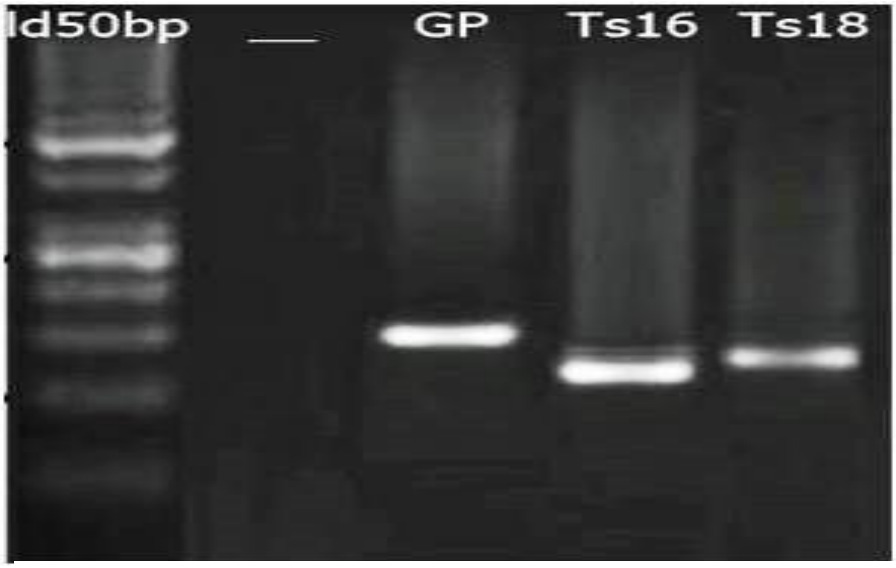


Figure 4.4 Reference Gel

Lane 1 showing molecular marker, Lane 2 showing negative control, Lane 3 GP, Lane 4 TS16 and Lane 5 Ts18

4.3 Sequence analysis of HPV16-L1 gene

In the current situation prophylactic vaccines are based on the self assembling property of the major capsid protein encoded by L1 gene. High cost vaccines prevents their use in most developing countries. Therefore we tried to screen HPV of various paraffin embedded cancer samples for L1 gene. For this we designed primers of L1 of whole gene of HPV16 but unable to optimized them afterwards we designed primers for partial sequence from them and detected the presence of L1 in only four samples as shown in figure 4.5. To confirm the authenticity of these four samples we eluted them and sent for sequence analysis.

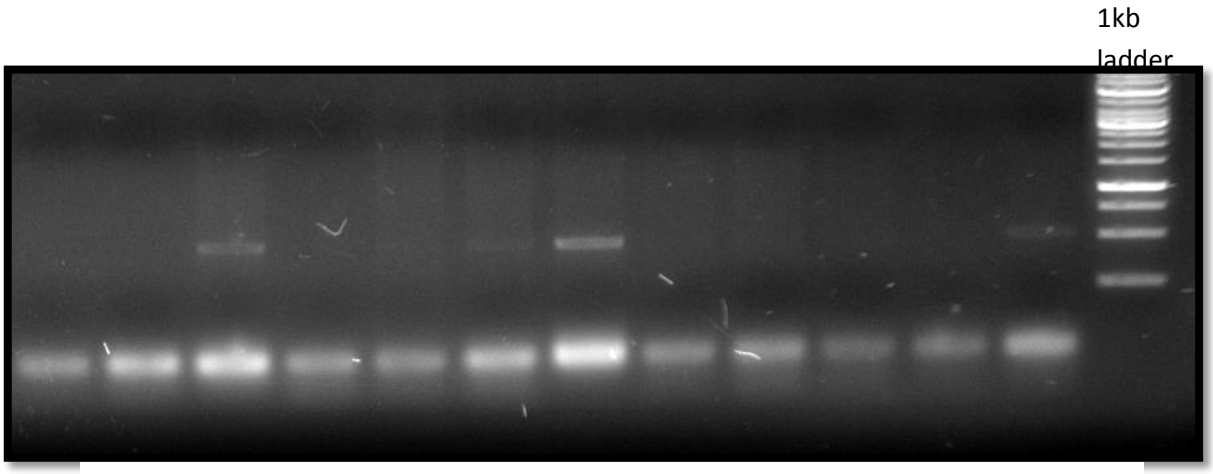


Figure 4.5 Showing PCR amplification of DNA using HPV-16L1 gene

Lane 1 on left side negative control and Lane 13 showing ladder of 1kb while Lane 2 to 12 of cervical cancer samples

4.1.2 Molecular characterization of HPV-16-L1

The phylogenetic tree of HPV16 L1 and L1 of other HPV genotypes were constructed using CLC workbench and shows that Pakistani variant was closest to that found in South African isolate of HPV-16-L1 as shown in figure 4.6.

4.6 In-silico analysis for epitope prediction

4.6.1 Consensus Sequence

Consensus sequence of HPV-16-L1 were obtained from sequences of Pakistan available in NCBI. The consensus sequence was then used to predict various antigenic epitopes within this protein. Probable antigenic protein value for L1 was identified by VaxiJen at 0.4 threshold level and the molecular weight of HPV-16-L1 was 56277.76 KDa by using Protein information database.

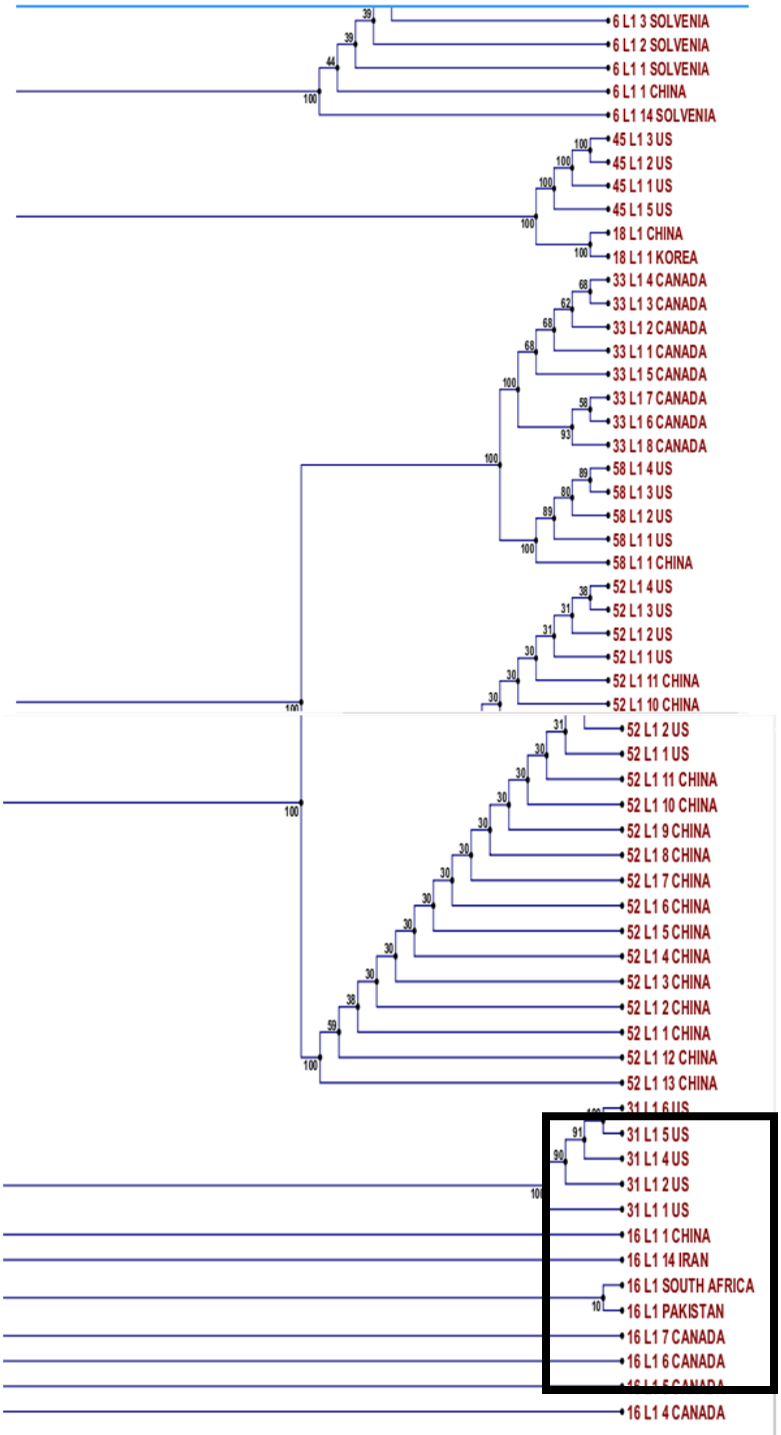


Figure 4.6: Phylogenetic analysis of HPV16 L1

4.6.2 B-cell Epitopes

A total of 10 B-cell epitopes were predicted by using IEDB. Among them epitope no. 1, 2, 3, 5, 9, 10 shows more than 50% conservation among HPV-16-L1 and other genotypes worldwide while epitope no. 4, 6, 7, 8 shows more than 90% conservation. On other side all these epitopes shows < 90% conservedness among HPV-16-L1 genotype as shown in Table 4.1.

4.6.3 T-cell Epitopes

About 13 epitopes predicted against class I MHC specific alleles by using online tool ProPred. Among these epitope no 7, 8, 12 were conserved <80% in HPV-16-L1 and other genotypes while all other epitopes show <40% conservance among all genotypes and while on the other side all epitopes except epitope no. 3 shows more than 90% conservedness among HPV-16-L1 as shown in Table 4.2.

Twenty two T-cell epitopes in HPV-16-L1 were predicted against class II MHC-specific alleles by ProPred-I. Epitopes 9 was found to be 90% conserved among HPV-16 and other genotypes and epitope no. 1, 6, 13, 19, 20 show <50% conservance among all other genotypes and HPV-16-L1, while epitope no. 2, 3,4, 5, 7, 8, 10, 11, 12, 14, 15, 16, 17and 18 showed <30% conservation among all genotypes. Epitope no. 1, 6, 7, 8, 9, 10,11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 shows <90% conserved only in HPV-16-L1 as shown in Table 4.3.

Epitopes	Names (location)	Asia HPV 16 (L1)		Asia HPV L1 (6,11,16,31,33,45,52.58)		World HPV 16 (L1)		World HPV L1 (6,11,16,31,33,45,52.58)	
		<50%	<90%	<50%	<90%	<50%	<90%	<50%	>90%
EYV	B1(26-28)		100%		96.6%		100%	62%	
PDPNKFGFPDTSFYNPDT	B2(78-95)		100%	80%			100%	50.9%	
VGRGQPLGVG	B3(107-116)		100%	80%			96.7%	83.6%	
GDMVDT	B4(198-203)		98.4%		98.8%		98.9%		99.5%
EPYG	B5(240-243)		100%		96.6%		100%	62%	
AQGHN	B6(316-320)		100%		100%		100%		100%
TTRS	B7(336-339)		100%		100%		100%		100%
E	B8(369)		100%		100%		100%		100%
SA	B9(456-457)		100%		96.6%		100%	62.0%	
KAK	B10(475-477)		98.4%	78.8%			98.9%	50.4%	

Table 4.1: B-cell Epitopes

Epitopes	Names	Asia HPV 16 (L1)		World HPV 16 (L1)		Asia HPV L1 (6,11,16,31,33,45,52.58)		World HPV L1 (6,11,16,31,33,45,52.58)	
		<50%	<90%	<50%	<90%	<50%	<90%	<20%	>90%
LPSEATVYL	1		97%		100%	70.6%		45.5%	
QYRVFRIHL	2	57.3%		67.3%		41.3%		29.8	
VEVGRGQPL	3	67%			95.7%	44.5%		46%	
SGHPLLNKL	4		97%		100%	70.6%		45%	
DDTENASAY	5		97%		98.9%	70.6%		44.5%	
LYIKGSGST	6		97%		100%	70.6%		47.8%	
YIKGSGST	7		97%		100%	70.6%		47.8%	
ENVLKEKFS	8		97%		100%	70.6%			85.7%
QFPLGRKFL	9		97%		98.9%		93%		85.7%
RKFLLQAGL	10		97%		97.9%	45.6%			53.0%
PKFTLGKRK	11		100%		98.9%		72.8%	44.0%	
FPLGRKKFL	12		97%		98.9 %		94.5%		85.7%
YHAGTSRLL	13		97%		96.8%	70.6%		43.6%	

Table 4.2: MHC I Epitopes

Epitopes	Names	Asia HPV 16 (L1)		World HPV 16 (L1)		Asia HPV L1 (6,11,16,31,33, 45,52.58)		World HPV L1 (6,11,16,31,33, 45,52.58)	
		>75%	<90%	>75%	<90%	>75%	<90%	>75%	<90%
VYLPPVPVS	1		100%		98.9%		97.7%	61.5%	
LQYRVFRIH	2	61.5%		67.7%		60%		30.2%	
YRVFRIHLP	3	61.5%		67.7%		60%		30.2%	
YRVFRIHLPDPN	4	61.5%		67,7%		60%		30.2%	
FRIHLPDPN	5	61.5%		67.7%		60%		30.2%	
LVPKVSG	6		100%		100%		97.7%	59.1%	
LELINTVIQ	7		96.91%		97.8%	70%		43.7%	
FFYLRREQM	8		100%		100%	72%		47.5%	
LFF	9		100%		100%				90.3%
LFFYLRREQM	10		100%		100%	72%		47.5%	
FVRHLFNRAG	11		100%		100%	72%		44.7%	
VRHLFNRAG	12		100%		100%	72%		44.7%	
LQFIFQLCK	13		100%		100%	88.8%		55.7%	
MTYIHSMNS	14		98.4%		97.8%	71%		43.7%	
IHSMNSTIL	15		98.4%		97.8%	71%		43.7%	
YRFVTSQAI	16		96.9%		97.8%	70%		43.7%	
YRFVTSQAIAC	17		98.4		98.7%	71%		44.2%	
FVTSQAIAC	18		96.9%		97.8%	70%		43.7%	
VNLKEKFSA	19		100%		100%	73.3%		50%	
LGRKFLLQA	20		100%		98.9%		94.4%	59.1%	
FLLQAGLKA	21	63.0%		56.9%		54.4%		34.6%	
LKAKPKFTL	22	63.0%		56.9%		45.5%		30.7%	

Table 4.3: MHC II Epitopes

To avoid the autoimmune response, all the predicted B-cell-binding and class I as well as II MHC-binding antigenic regions was analyzed for homology with human proteome and no epitope was found to be homologous with human proteome.

CHAPTER 5

DISCUSSIONS

Cervical cancer, the cancer of cervix arose when unusual growth of cells occur in cervix. Cervical cancer is among the seventh most common cancer globally in women (Stewart & Wild, 2014). Cervical cancer screening is acknowledged as currently the most effective approach for cervical cancer control. Observational data supports the fact that regular cytology based cervical screening programs with the Pap test have been effective in reducing both the incidence and cervical cancer-related mortality rate. The human papillomavirus (HPV) infection is one of the most prevalent sexually transmitted disease (Koutsky et al., 1997). HPV infection is a necessary cause of cervical cancer, but it is not a sufficient cause. Promiscuous sexual behaviour has been clearly established as a predominant risk factor for acquiring genital HPV infection. The HPV especially their high risk types HPV-16 and HPV-18 are the causative agents of almost all cervical cancer patients. However HPV-16 is the most prevalent one because of its higher prevalence than HPV-18. In the developed countries HPV screening through PAP tests and cytological analysis practiced effectively, which help in the early detection of any precancerous or cancerous lesion that can lead to malignant cancer and thus helps in the good clinical outcome. In Pakistan and other developing countries the general lack of public awareness and understanding of HPV infection and its cancer causing ability, its vaccination and screening.

A large number of studies are evidence of HPV as an aetiological cause of all cervical cancers globally (Walboomers et al., 1999). Most the studies are in agreement that HPV-

16 and 18 are the most prevalent HPV subtypes (Mukoz et al., 1992 ; Bosch et al., 1995). Overall percentage of GP positive cancer samples was 50% however with TS primers a higher percentage of cancers samples were found to be HPV positive. The main reason of this difference could be the advanced stages of cancers because in advance stage of cancer the region of L1 deleted in some cases.

HPV genomic expression is very much regulated and it is therefore, that the L1 gene which is the most immunogenic gene has been reported to be deleted in the advanced stages of cancers. Therefore, the use of GP primers is not enough for the accurate detection of HPV. In recent times a lot of controversies have taken place regarding the role of HPV in cancers other than cervical cancers. HPV has been found in anogenital cancers, breast cancer and lungs cancer in addition to cervical cancer but still the aetiological role remains to be controversial because of different results in these different studies. In order to estimate the HPV related disease burden especially pertaining to the cancers other than cervix we screened different cancer samples for the presence of HPV. We took samples of different cancers to check the prevalence of HPV. In recent years, evidence indicates that HPV may also have a role in breast cancer. The involvement of the virus in breast cancer remains questionable. Earlier reports have shown that HPV DNA was detected in breast cancer specimens and the prevalence of HPV positive breast cancer vary from one study to another (Lawson et al., 2006). A recent study in India established no correlation between HPV and BC.ref High risk types 16, 18 and 33 of HPV have been screened in breast cancers patients from a wide group of different

populations using different techniques and the study shows that oncogenic characteristics of HPV associated breast cancer related to HPV associated cervical cancer (Lawson et al., 2006). To confirm this we include breast cancer patients in our study and amplify the DNA to check the prevalence of HPV by using different primers such as GP, Ts16 and Ts18 and predicted the prevalence of HPV in 7% of cases using GP whereas 16% of cases were detected with Ts16 and none of the cancer patients were HPV18 positive. Our study concluded that these viruses can be implicated in the genesis of breast carcinoma. Further analyses are in progress to extend these preliminary results to a greater number of specimens.

The presence of HPV in endometroid cancer has also been reported, although less commonly. In different group of studies by using different techniques investigated the role of HPV in endometroid carcinoma and find out positive association of HPV in endometroid cancer (Fujita et al., 1995) to conclude this, we take endometroid cancer patients for HPV detection using the same primers as in case of breast cancer and found positive association of HPV in 50% of cases with Ts16 and no association of HPV18. Likewise in the case of prostate cancer only 16.6% of cases showed positive association with Ts16 and again no association with HPV18. Therefore, these mixed results are consistent with the present controversial landscape on the role of HPV infection in prostate carcinogenesis. To find out the solution of this dispute about the role of HPV in prostate cancer their needs additional research to give the answer.

Lungs cancer is leading cause of death worldwide both in males and females. In addition to smoking other environmental factors also responsible for lungs cancer. Main aetiological factor responsible for lungs cancer is controversial. Human papillomavirus (HPV), known to be an etiological agent for genital cancers, has been suggested also to be a possible contributory agent for lungs cancer. Different studies performed to investigate the role of HPV in lungs cancer and concluded that HPV is second leading cause of lungs cancer after smoking. A lot of studies confirmed the presence of HPV in lungs cancer but we are unable to find significant association as we predicted only 11% of cases with positive association between HPV and lungs carcinoma patients because of small size of lungs cancer patients.

In recent years controversial role of HPV in rectal cancer is increasing but defined results are still undergoing. In earlier times group of studies shows the prevalence of HPV in rectal cancer significantly. Among these cases, HPV frequency decreases with increasing severity of the disease yet, it remains to be seen whether the association of HPV with the earlier stages of colon adenocarcinoma is a reflection of a true absence of viral infection in the advanced stages or simply a biologic consequence of viral integration(Perez et al., 2005). In our study we found 31% of cases showed positive association with Ts16 and only 7% positive association with HPV18. In addition to these a large number of studies on other anogenital cancers such as vaginal cancer and ovarian cancer carried out and concluded with the findings that HPV involve in the pathogenesis of these cancer. In our study we found 71.4% in ovarian cancer 66.6% vaginal cancers which shows the strong

association of HPV-16 with these cancers. Among these different cancer patients which are positive all comes under the HPV type 16. The only exception is that HPV18 is responsible for 7% in rectal cancer. Prevalence of all these different malignancies showed that HPV may play an important role in the pathogenesis of these cancer but still need to devise systems of research which find out the exact role of HPV in these cancers. As in the case of prevalence of HPV in cervical cancer sensitivity of GP is less than type specific primers of HPV. According to the literature review some studies showed that as GP targeted the conserved region of L1 so in some cases L1 region deleted that's why unable to get more accurate result.

Positive association of HPV in cervical cancer and other anogenital and non-anogenital cancers requires effective treatment so as to eradicate the infection of virus. For this vaccines should be a good effort such as Gardasil and Cervarix which were designed against different HPV types but due to their limitations not applicable widely such as high cost of these vaccines and designed aginst some types of HPV so, their need to find or design a particular vaccine which is cost effective and should be effective against all subtypes either low risk types or high risk types.

To answer this issue we have analyzed HPV-16-L1 gene by using different methods like in-silico analysis and amplification. Amplification of this gene in cervical cancer patients using partial sequence of L1 but unable to amplify all samples although positive samples are eluted and then sent for sequencing in order to analyze the authenticity.

To understand the evolutionary and genetic link of Pakistani variants of L1 with the L1 variants of the world alignment of L1 available on NCBI from same genotype and different genotypes around the world was carried out and using this alignment phylogenetic tree was constructed. Phylogenetic analysis is essential not only for epidemiology purposes but also for the determination of epidemics and endemics so that immunization programs can be indicated. It was seen that Pakistani variant was closest to that found in South African isolate.

As there is an utmost need of proper subunit vaccine which drops the incident of cervical cancer we have analyze the in silico analysis of L1 gene and find out 10 B-cell epitopes among them four epitopes showed more than 90% conservedness among all genotypes. 35 T-cell epitopes by using online tools among them about five epitopes showed more than 80% conservedness among all genotypes which are targeted to form peptide vaccine which is the need of nature. This approach should be beneficial in all the way because it occupy all the genotypes and hence protect against all genotypes whether high risk or low risk

CHAPTER 6

CONCLUSIONS

We have optimized PCR based detection of HPV and its genotypes 16 and 18. About 50% of cervical cancer patients shows positive association using GP and when both type specific primers used 75% of cases were detected for HPV which shows that type specific primers are more sensitive as compared to GP. Single infection of HPV-16 involved in 64.5% cases and about 27.7 % of cases positive for HPV-18 and in 21% of cases co-infections of both types of HPV. In addition to that detection of HPV in other anogenital cancers, breast cancer and lungs cancer showed the same manner of using different primers, among them HPV-16 is the most prevalent one while only one cancer patient shows involvement of HPV-18 which belongs to rectal cancer. We have proposed the idea of subunit vaccine in order to minimize the limitation we have analyzed HPV-16 L1 gene by using different methods like in-silico analysis and amplification. Phylogenetic tree of single variant of Pakistan HPV-16-L1 showed correlation with that of South Africa. Amplification of 16-L1 unable to amplify all cervical samples due to its expression in early stages of cancer. In-silico analysis of L1 gene using online tools find out 10 B-cell epitopes out of which four epitopes shows more than 90% conservedness and among the 35 T-cell epitopes five epitopes shows higher conservedness which are targeted to form peptide vaccine which is the need of nature.

CHAPTER 7

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