INVESTIGATING PREVALENCE AND GENOTYPING OF HIGH-RISK HPV IN PROSTATE CANCER PATIENTS FROM PAKISTAN

By

Ayesha Irfan

(Registration No: 00000400056)

Supervisor: Dr. Saira Justin

Department of Biomedicine

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

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(2024)

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Supervisor: Dr. Saira Justin

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

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Signature: Dr. Saira Justin, Assistant Professor, Deptt of Healthcare Biotechnology, Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Name of Supervisor: Dr. Saira Justin

Date: 23/8/24

Signature (HOD): Dr. Hussain Mustafa Wahedi, Head of Department (HoD), Deptt of Healthcare Biotechnology, Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 02/09/2024

Signature (Dean/ Principal): Prof. Dr. Muhammad Asghar, Principal & Dean, Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad

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Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

(Supervisor)

Dr. Saira Justin

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Examination Committee Members

1. Name: Dr. Aneela Javed Signature: _____

2. Name: Dr. Rumeza Hanif Signature: _____

3. Name: Dr. Naureen Ehsan Ilahi Signature: _____

Supervisor's name: Dr. Saira Justin Signature: _____

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Date: 23/8/24

02/09/2024
Date

Dr. Hussain Mustafa Waheed
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad
Head of Department

COUNTERSINGED

03/09/2024
Date

Prof. Dr. Muhammad Asghar
Principal & Dean
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST, Islamabad
Dean/Principal

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DEDICATION

With profound gratitude, I dedicate this dissertation to Almighty ALLAH for His countless blessings. I also wish to honor the remarkable men in my life who have steadfastly believed in and supported me. This work is dedicated to my late grandfather, Lt. Col. Raja Muhammad Ikram Ullah Khan, my father, Mr. Irfan Ahmed, and my two incredible brothers, Mr. Sehel Sohail and Mr. Mustafa Irfan. Their constant encouragement to dream big, their unwavering support, and the invaluable life lessons they've imparted—reminding me that neither worries nor setbacks are permanent—are deeply cherished. I am forever grateful to have them in my life.

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Ayesha Irfan

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List of Abbreviations

%	Percentage
µl	Micro-liter
µm	Micro-meter
°C	Degree celsius
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide
β-globin	Beta-globin
CDK	Cyclin-dependent kinase
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
E6AP	E6 associated protein
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FFPE	Formalin-fixed paraffin-embedded
g	Gram
HDAC	Histone deacetylases
HPV	Human papillomavirus
HR-HPV	High-risk human papillomavirus
IARC	International Agency for Research on Cancer
IPA	Isopropyl alcohol
ISUP	International Society of Urologic Pathologists
kb	Kilobase
L	Liter
LCR	Long control region
LR-HPV	Low-risk human papillomavirus
M	Molar
mg	Milli-gram
$MgCl_2$	Magnesium chloride

NaCl	Sodium chloride
NF H_2O	Nuclease free water
ng	Nanogram
NWSM	Department of Pathology, Northwest School of Medicine
ORF	Open reading frames
PCR	Polymerase chain reaction
PML-NBs	Promyelocytic leukemia nuclear bodies
pRB	Retinoblastoma protein
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate ethylenediaminetetraacetic acid
vol	Volume
WHO	World Health Organization

Abstract

Human papillomavirus (HPV) attributable cancers are more pronounced in low-middle-income countries (LMICs), where it presents a significant challenge due to limited access to screening and vaccination programs, coupled with inadequate hygiene conditions. In Pakistan, the etiological association of HPV with anogenital and urologic cancers remains under-researched. Globally, the association of HPV with prostate cancer remains elusive, and to date, no study has been conducted on this aspect in Pakistan. Therefore, this study aims to explore the link between HPV and the development of prostate cancer in the Pakistani population.

This study included 50 men with primary prostate cancer, clinically confirmed through Hematoxylin and Eosin (H&E) staining and Immunohistochemistry (IHC) staining. Formalin-fixed paraffin-embedded (FFPE) prostate tissue biopsies, along with histopathological data, and clinicopathological data, were collected after informed consent. DNA was extracted and processed for HPV detection using L1 consensus primers, via conventional PCR.

Histopathological and clinicopathological analysis revealed that in Pakistan, prostatic acinar adenocarcinoma was the only type of prostate cancer, with 64% (32/50) of patients having low to moderate grade cancer and 42% (21/50) patients having Gleason score of 7. The average age of patients was 65 years. All patients were married with 92% (46/50) belonging to urban areas. All patients had PSA levels above 4.0 ng/ml. Among co-morbidities, 78% (39/50) patients had lower urinary tract symptoms (LUTS) followed by benign prostate enlargement (BPH) in 24% (12/50) patients, making them the most prevalent. Regarding contemporary lifestyle choices, 52% (26/50) of patients had an active lifestyle and 74% (37/50) were tobacco abstainers. HPV detection via PCR did not identify the virus in any patient and thus the study did not establish a causal link between HPV and prostate cancer.

To gain a deeper understanding of the link between HPV and prostate cancer, more comprehensive research is needed. This should involve larger and more diverse sample

sizes, including cases of benign prostatic hyperplasia and samples from different regions of Pakistan, consideration of additional risk factors such as sexual history, and the use of techniques beyond molecular analysis, such as immunohistochemistry (IHC) for protein detection and sequencing.

Keywords: HPV; HPV-16; HPV-18; prostate cancer; STD's

Graphical Abstract

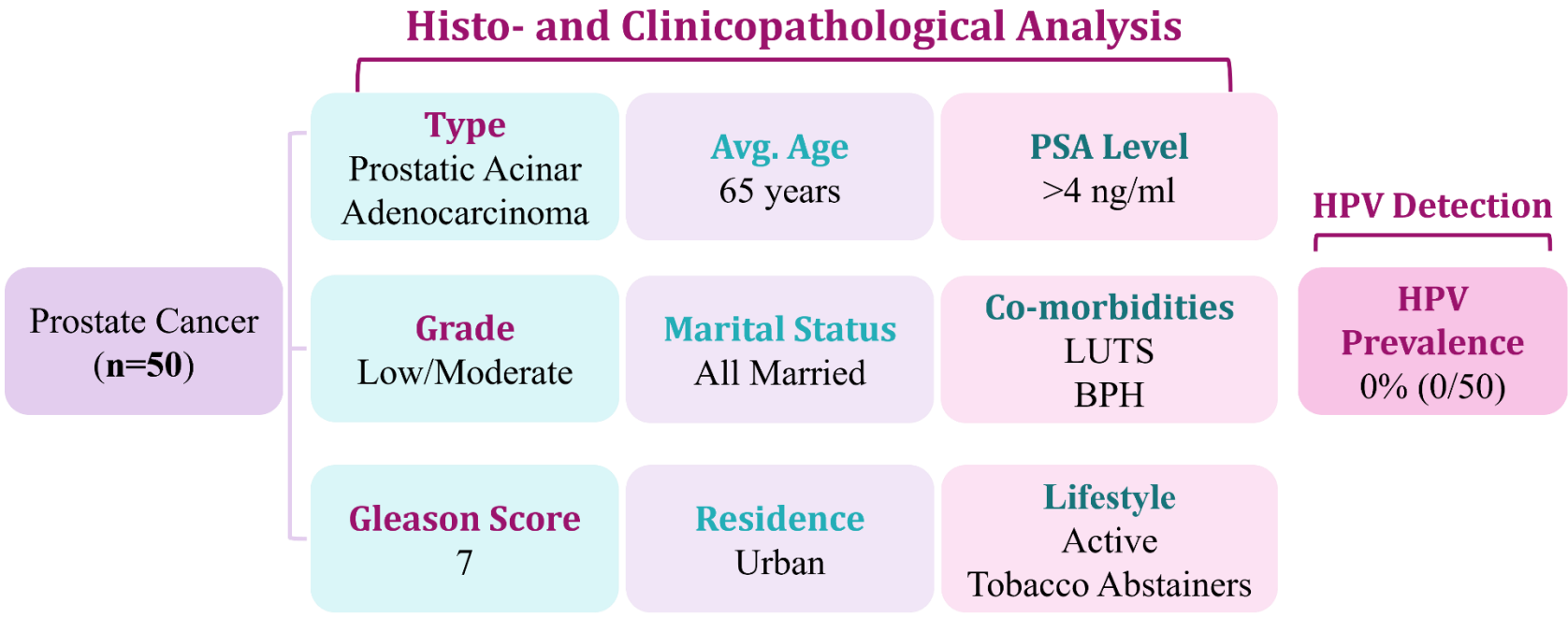


Figure 1: Graphical Abstract

Chapter 1

Introduction

Sexually Transmitted Diseases (STDs) or Sexually Transmitted Infections (STIs) have been recognized as major public health concerns across the globe. Suspected culprits of this medical burden have been traced, including bacteria, viruses, and other parasites, that are known to cause around 30 sexually transmitted infections. WHO has reported that every day 1 million STIs are acquired across the globe and 347 million infections are acquired annually (Ağaçfidan & Kohl, 1999; De Schryver & Meheus, 1990)

The eminent route of their transmission is the sexual route, however poor hygiene and poor living conditions are also known to contribute to their spread. STDs are known to infect the lining and organs of the reproductive tract (Da Ros & da Silva Schmitt, 2008). These infections remain asymptomatic over a while. In addition to this, considering them as a social stigma people avoid taking medical advice even if the symptoms are visible. As a result of prolonged untreated infection, inflammation migrates to the pelvic region and other organs near the reproductive tract.

One of the major viral causes of STDs across the globe is Human papillomavirus (HPV). Successful immune strategies clear infection caused by the tiny virus of the host immune system. However, if the immune system fails to clear the infection and the infection persists over a long period, the virus celebrates its victory by causing malignancy in the anogenital tract of the host.

Around 200 genotypes of the virus have been identified. The genotypes of the virus have been classified as high-risk genotypes and low-risk genotypes (Burd, 2003). Low-risk genotypes have cutaneous targets, including the skin of hands and feet. The high-risk genotypes have mucosal targets targeting the lining of the throat, respiratory tract, and anogenital tract (Egawa et al., 2015). Due to the epithelial tropism of HPV, squamous cell

carcinoma is the most prevalent histologic type of cancer caused by HPV, and squamous intra-epithelial lesions in the case of warts caused by HPV (Stanley, 2010). It has been reported by WHO in 2023 that HPV infection causes about 5% of all cancers worldwide, with an estimated 625 600 women and 69 400 men getting HPV-related cancer each year (Moscicki, 2007).

HPV is the most common cause of female cervical cancer which is the 3rd most common female cancer around the globe and 2nd most leading cause of death in female after breast cancer (Carter et al., 2011).

HPV has been identified as the underlying cause of 91% of cervical cancer cases, 91% anal cancers, 65% of vulvar cancers, 75% of vaginal cancers, 63% of penile cancers, and 70% of oropharyngeal cancers (Stanley, 2010).

In addition to these scientists have been working to trace the suspicion of HPV in other malignancies of the anogenital tract including bladder, renal, and prostate cancer. However, no irrefutable conclusion has been yet stated by the scientists if HPV is also involved in malignancies of other organs of the anogenital tract and urinary tract.

1.2 Aims and Objectives

Aim of Study

The study aims to establish the etiological association of HPV genotypes with prostate cancer patients in Pakistan.

Objectives

1. Establishing an etiological association between HPV and prostate cancer.
2. Genotyping of high-risk HPV-16 & HPV-18.

Chapter 2

Literature Review

2.1 HPV Genome and Structure

HPV is said to be the most common culprit of sexually transmitted infections. The papillomaviruses comprise tiny non-enveloped icosahedral DNA viruses with 55nm in diameter. HPV is epitheliotropic in nature and specifically infects the epithelial cells of the skin and mucous membrane of the mouth, hands, feet, and other organs of the anogenital tract. The genome of HPV is double-stranded circular and is approximately 8kb in size (Schwarz et al., 1983). The genome of the HPV is divided into three major regions: early, late, and non-coding. The early (E) region of the genome constitutes half (50%) of the total genome and encodes six open reading frames that translate six different E-proteins of the virus. Furthermore, the late region (L) of the HPV covers around 40% of the total viral genome and encodes for two structural proteins L1 and L2, capsid proteins (Vashisht et al., 2019). The non-coding region of the HPV accounts for only 10% of the total viral genome and does not encode for any protein however bears the origin of replication and is also the site for binding of numerous replication factors (Zheng & Baker, 2006). The Figure 2 presents the genomic organization of HPV.

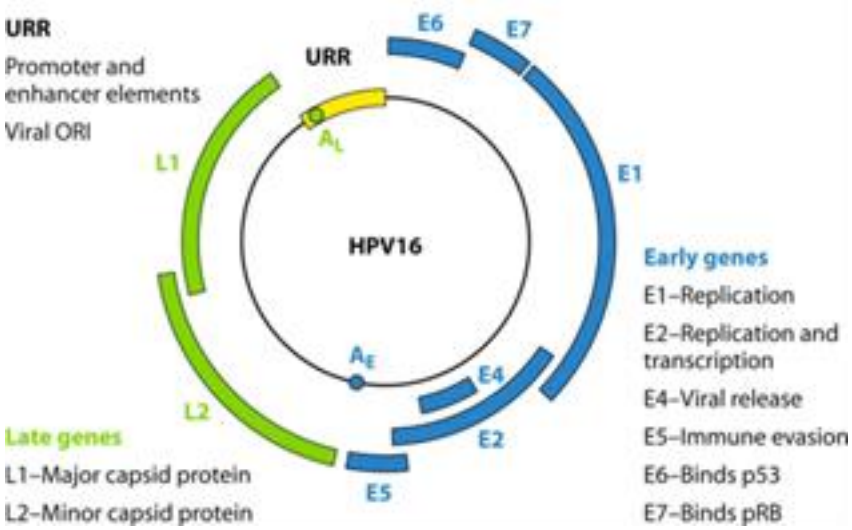


Figure 2: Genome organization of HPV (Shrestha, 2022)

The Table 1 represents the proteins of HPV along with their functions in the viral life cycle (Graham, 2010):

Table 1: Functions of HPV Proteins

Protein	**Function**
E1	Genome replication
E2	Genome replication, regulation of cellular genes, regulation of cell cycle and apoptosis
E4	Modification of cellular cytokeratin networking, cell cycle arrest
E5	Controls cell growth and cell differentiation
E6	Inhibits apoptosis and cellular differentiation, regulates cell shape, polarity, and mobility
E7	Controls cell cycle
L1	Major capsid protein
L2	Minor capsid protein, recruits L1, involved in viral assembly

2.2 HPV Genotypes

Infections caused by HPV are considered the most common sexually transmitted disease across the globe and target both men and women. Approximately 200 genotypes of the HPV have been identified based on DNA sequencing and genomic differences. The virus is known to infect the basal epithelial skin and the inner lining of the tissues. Therefore, the virus is said to be epitheliotropic and is known to cause lesions and warts on the skin and malignancies of the anogenital tract. Depending on the tropism and structural variations among HPV genotypes, they are categorized into four genera, Alpha, Nu/Mu, Beta, Gamma. The Alpha HPV genotypes are further classified as high-risk HPV and low-risk HPV depending on the potential to cause carcinomas or precursor lesions. High-risk HPV genotypes are associated with the development of anogenital and oropharyngeal carcinomas (Harari et al., 2014). However, the low-risk HPV genotypes are associated with the formation of cutaneous lesions and warts. The low-risk HPV genotypes (6, 11, 42, 43, 44, 54 and 70) are associated with the formation of low- and high-grade lesions and benign tumors. Furthermore, the high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) are involved in low- and high-grade lesions and anogenital carcinomas. Both high-risk HPV and low-risk HPV genotypes infect the mucosal and genital sites.

The genotypes (1, 4, 5, 8, 17, 63, 96, 88 and 135) that fall in the other three genera infect the cutaneous sites and are involved in the formation of skin, palmar, and plantar warts (Burd, 2003).

2.3 Viral Oncoproteins of HPV

Three proteins from the early region E5, E6, and E7 are known to have oncogenic potential, which are discussed below:

2.3.1 E5

The protein has a prime role in differentiation-dependent stages of the viral cell cycle, however, E5 protein is found to be weakly oncogenic as compared to the E6 and E7 proteins. The literature suggests that E5 may have its role in enhancing the function of E6 and E7 and thus is not directly involved in oncogenesis. E5 protein binds to the growth factor receptor and enhances cell growth and proliferation making the environment more suitable for potential oncogenesis (Scarth et al.,

2021). Furthermore, it has also been demonstrated that the E5 protein has the potential to prompt the functioning of protooncogene c-Jun leads to uncontrolled cell proliferation (Chen et al., 2007).

In addition, several studies have also demonstrated the E5-mediated activation of EGFR, which is a central factor in the development of hyperplasia, and cellular transformation and is also involved in uncontrolled cell cycle progression during the keratinocyte differentiation (Genther Williams et al., 2005).

The E5 protein of HPV is also known to have a significant role in one of the most pivotal hallmarks of oncogenesis, inhibition of apoptosis. The HPV induces stress on the infected cells and manages to abrogate the cell cycle checkpoints. The protein is known to stimulate the proteasomal degradation of the pro-apoptotic Bcl-2 family member BAX and also down regulates the expression of the Fas receptor (Oh et al., 2010).

The E5 protein also sets the strategy to evade the of impeding the expression of several critical immune mechanisms that detect any threat for the body therefore contributing to potential oncogenesis, including MHC-I, MHC-II and CD1d (Zhang et al., 2003).

2.3.2 E6

The E6 protein of HPV is known as the most significant protein in the development of HPV-induced carcinomas. E7 protein is known to have its role in the initial settings of cancer development, however E6 is known to have its role in the acceleration of tumor development. The most eminently known oncogenic strategy of the E6 protein is the degradation of the p53 tumor suppression gene, which leads to the blocking of pro-apoptotic signals (Scarth et al., 2021). Under normal conditions, p53 protein is known to induce pro-apoptotic activities including cellular senescence, cell arrest and apoptosis. HPV-infected cells are stressed causing phosphorylation of p53 followed by proteasomal degradation of p53 resulting in disrupted cell cycle. Direct binding of the E6 protein to p53 has been reported, along with sequestration of p53 in cytoplasm, all these events lead to hinder the binding of p53 with DNA, therefore abrogating the pro-apoptotic signals and inducing cell proliferation (Scheffner et al., 1990).

In addition to this E6 protein also targets other cellular mechanisms to avoid normal cell cycle regulation. Binding to Bcl-2 homolog BAK is one of the notable goals of E6 protein in this regard. Furthermore, the E6 protein immortalizes the cell that contributes to oncogenesis. The E6 protein enhances the activity of the telomerase, therefore resulting in the lengthening of the telomeres and indefinite cell proliferation (Mittal & Banks, 2017). E6 protein successfully mimics several cellular pathways to achieve its oncogenic role. The notch signaling pathway is one of the most highly conserved pathways that play a crucial role in cell differentiation, proliferation, and the decision of cell fate. Upregulation of the Notch-1 receptor has been reported in the keratinocytes of cervical cancer with HPV infection thus contributing to cervical carcinogenesis (Boulet et al., 2007).

PI3K/AKT/mTOR pathways are among the most critically regulated cellular pathways, that have their roles in cell metabolism, motility, and proliferation and are commonly observed to be dysregulated in most human cancers. The E6 protein is known to inhibit the functioning of PI3K and promote the PTEN pathway and also has a role in increased phosphorylation of PI3K/AKT leading to cancer progression (Porta et al., 2014).

E6 protein also impairs the activation of the non-receptor tyrosine kinase 2 (TYK2) therefore turning down the JAK/STAT signaling and is also known to interact with components of the innate immune system, preventing the expression of interferons and thereby making the scrutiny of viral threat a challenge for the immune system (Ronco et al., 1998).

2.3.3 E7

The E7 protein of HPV is known as a major transforming protein and its expression plays a considerable role in cancer progression. The oncogenic characteristic feature of the E7 protein is dysregulating the G1/S phase transition, by interfering with the activity of the E2F transcription factor and enhancing cell proliferation (Scarth et al., 2021). E7 protein binds to the pRb protein and releases the E2F transcription factor enabling the activation of genes related to E2F, that have a role in the progression of the cell cycle, enhancing the re-entry into S-phase in differentiating keratinocytes. E7 protein has the capability of targeting Rb proteins by causing their proteasomal degradation (Darnell et al., 2007). In addition to this

E7 protein also impairs cellular differentiation, which is done by manipulating the TGFβ pathway, and also affects cellular transformation. E7 protein also interacts with the host immune response to ensure the successful implementation of its oncogenic activity. It has been reported that host PRR is repressed in the HPV+ cervical cancer cells. The E7 protein is also responsible for the epigenetic silencing of dsDNA Toll-like receptor 9 by recruiting histone deacetylases that prevent the product of interferons. NFκB also plays a significant role in host anti-viral immune policy. E7 protein lowers the expression of NFκB signaling pathway in the infected keratinocytes (Richards et al., 2015). Studies of animal models have also presented that the E7 protein is also responsible for inducing an immunosuppressive environment where is activity of cytotoxic T lymphocyte is highly compromised (Bergot et al., 2014).

The E7 protein is also known to induce the genomic instability as it hampers the pRb pathway and affects the activity of E2F transcription factor leading to uncontrolled proliferation which ultimately induces replication stress and genomic instability escalating the oncogenic potential of the E7 protein (Bester et al., 2011).

2.4 HPV Infection and Lifecycle

The pathogenesis of the virus is linked to cellular differentiation of the epithelial cells (stratified epithelia of the oral cavity and anogenital tract), as the virus is strictly epitheliotropic. Infection in the body occurs via wounds or micro-wounds that make the basal epithelial cells of the target organs vulnerable to viral attacks involving certain receptors. The viral genome is established, and nuclear plasmid and early genes are expressed at low levels in the basal cells which depends on the expression of certain viral genes including E1, E2, and E6. HPV infects the stratified epithelia and thus viral genes are expressed in the nucleus of the cells and the virus critically maintains its copy number in the undifferentiated cells (Graham, 2017). Two eminent viral proteins E1 and E2 responsibly ensure the replication of the virus as they are notable transcription factors of the virus (Humans, 2007). Figure 3 represents lifecycle of HPV.

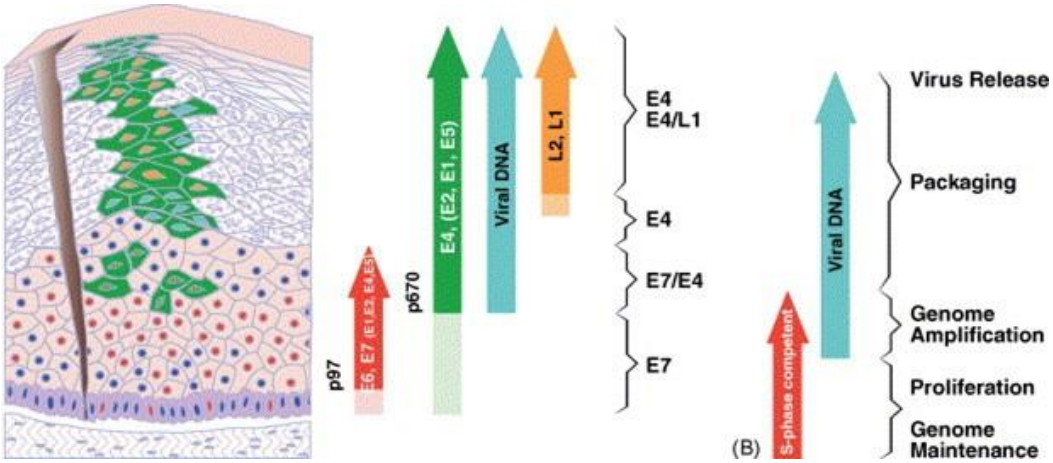


Figure 3: Lifecycle of HPV (Doorbar, 2005)

In normal tissues, the daughter loses their contact as they move towards the supra-basal compartment and the DNA synthesis is halted marked by terminal differentiation. Contrarily, the cells infected with HPV express E7 protein in which the migrating daughter continues DNA synthesis and expresses significant levels of cell proliferation markers (Doorbar, 2005). Abrogation of cell cycle check points and turn down of pRb pathways by the E7 protein of HPV is considered as its major hallmark towards oncogenesis. E6 and E7 proteins contribute to the immortalization of the cells as well as their uncontrolled ability to replication by continuing their cell cycle (Graham, 2010). The uncontrolled division of the cells in differentiating epithelial gives the physical appearance and clinical manifestation as a wart. Eventually when the differentiation reaches the granular stage of the epithelial cells L1 and L2 proteins are expressed and thus encapsulate the latest forming progeny of virus (Boulet et al., 2007; Hebner & Laimins, 2006).

2.5 Carcinomas Induced by HPV

Persistent infection of HPV can cause carcinogenesis depending upon the site of tropism of the virus. The virus is known to mainly cause six cancers, cervical, anal, penile, oropharyngeal, vulvar and vaginal. The virus is known to affect the epithelial cells and thin flat squamous cells in the internal lining of the organs thus most of malignancies caused by HPV are squamous cell carcinomas (Zaravinos, 2014). 90% of cases of anal, 63% of

penile, 75% vaginal, 69% vulvar cancers are known to be caused by HPV. In addition to this all the cases of cervical cancer all across globe are said to be caused by HPV. In addition to this 70% oropharyngeal cancers are caused by HPV, which include malignancies of head and neck especially oral cavity and pharynx (Devaraj et al., 2003; Stanley, 2010).

2.6 Prostate Cancer

Prostate cancer is the uncontrolled growth of the cells in the prostate gland. The prostate gland is part of the male reproductive system, where the gland functions to produce seminal fluid, nourish the sperm, and aid the transport of sperm. Prostate cancer is one of the most common cancers among men across the globe and is treatable if diagnosed at early stages. Some of the common symptoms experienced by patients of prostate cancer are trouble in urination, blood in urine or semen, weight loss, and erectile dysfunction (Swallow & Kirby, 2008).

2.7 Epidemiology of Prostate Cancer

Prostate cancer is among the fourth most diagnosed across the globe and the eighth most common leading cause of mortalities worldwide. According to Globocan, 1,467,854 cases of prostate cancer were diagnosed, and 397,430 mortalities were reported in 2022. The incidence and mortality of the diagnosis of prostate cancer depend on diagnosing techniques and the age of the individuals (Grönberg, 2003).

African American men are more prone to the development of prostate cancer as well as develop more aggressive types of prostate cancer. Different studies in this regard have been conducted to unveil the major risk factors of prostate cancer that make African American men more vulnerable (Crawford, 2003).

According to the Pakistan National Cancer Register, prostate cancer is the second most common cancer in Pakistani men. In addition to these different studies have been conducted to find the burden of prostate cancer region-wise revealing that the incidence of prostate cancer is highest in KPK, 8.29%. This is followed by Punjab 8.09% and then Sindh, 3.3%. The differences in the incidence rates can be because of the socioeconomic conditions and sociocultural practices in different populations and depend on the screening methods (Akhtar et al., 2023).

2.8 Risk Factors of Prostate Cancer

Certain factors are recognized as crucial risk factors and have their role in the development of prostate cancer. The well-established risk factors of prostate cancer include advanced age, ethnicity, genetic factors, and family history. However other factors like diet, physical activity, obesity, and exposure to environmental chemicals and radiation and sexually transmitted infections (STI's) are also under study.

2.9 Possible Pre-Cancerous Conditions of the Prostate

Findings of the research have shown that certain possible conditions arise before the development of prostate cancer.

2.9.1 Prostatic Intra-epithelial Neoplasia (PIN)

The condition is characterized by the neoplastic growth of the epithelial cells, prominent nuclei are observed along with existing ducts. Pathologists have assigned four major patterns of cells to PIN, which include tufting, micropapillary, cribriform, and flat. The condition is characterized as a precursor of prostate cancer (Bostwick & Qian, 2004) (Brawer, 2005).

2.9.2 Prostate Inflammatory Atrophy (PIA)

In the benign prostate tissue, the cells of the gland that lie in close vicinity to chronic inflammation, have proliferated and attained the morphology of atrophy. These sites of the benign prostate are termed prostate inflammatory atrophy and are also considered early indicators of prostate cancer in most cases (Celma et al., 2014).

2.10 Types of Prostate Cancer

2.10.1 Adenocarcinoma

This type of cancer begins in the glandular region of the prostate, the gland cells that produce the prostate fluid which combines semen and sperm. Prostatic adenocarcinoma can be of two different types depending on the location of the cancerous cells.

Acinar

Abnormal cancerous cell growth is observed in the gland cells that line the prostate.

Ductal

This type of cancer begins in the cells that line the ducts of the prostate gland.

2.10.2 Small Cell Carcinoma

This is a rare type of prostate cancer and constitutes for 1% of cases. This type of prostate cancer occurs in the neuroendocrine cells of the prostate cancer and thereby affect the nerves and hormonal coordination of the gland. This type of prostate cancer is quite aggressive and quickly spreads to other parts of the body.

2.10.3 Transitional Cell Carcinoma

This is a rare type of prostate carcinoma that develops in the lining of the urinary tract and specifically urinary bladder. The tumor then tends to expand and invade the prostate gland and is difficult to distinguish from adenocarcinoma. This kind of prostate cancer is more aggressive than adenocarcinoma and has limited diagnosis and treatment options.

2.10.4 Sarcoma:

Sarcomas develop in the connective tissue cells of the prostate gland. This type of prostate cancer is uncommon, and more aggressive, however has limited treatment options.

2.11 Treatments for Prostate Cancer

2.11.1 Surgery

Radical prostatectomy is one of the most widely practiced surgery for the treatment of prostate cancer as this involves the removal of the entire prostate gland. (Sebesta & Anderson, 2017).

2.11.2 Radiation Therapy

There are several types of radiation used for the treatment of prostate cancer. External beam radiation therapy is used for the treatment at the initial stages, in

which the radiation from outside the body from a machine are bombarded at the targeted region. In addition to this three-dimensional conformal radiation therapy is also used that is a computer guided technique. (Podder et al., 2018).

2.11.3 Cryotherapy

The technique involves the use of very cold temperatures to freeze and kill the cancer cells in the prostate. This treatment option is usually considered after radiotherapy. The surgeon gives local or general anesthesia to the patient which is followed by intruding several needles via the anus or scrotum to access the prostate and then cold gases are passed through to kill the cancer cells in the prostate (Shinohara, 2003).

2.11.4 Hormone Therapy

High levels of androgens are considered the prime reason for prostate cancer. The two major androgens in the body are testosterone and dihydrotestosterone, which are usually produced by the testicles and adrenal glands. However, prostate cancer cells can also produce androgens that help them nourish and proliferate. This treatment option for prostate cancer involves surgery as well as the use of drugs (Desai et al., 2021).

2.11.5 Chemotherapy

Chemotherapy is used as a treatment option in advanced and aggressive cases of prostate cancer. The chemotherapy is usually accompanied by other treatment options which in most cases is hormonal therapy. The most used chemo drugs in case of treatment of prostate cancer, Docetaxel, Cabazitaxel, Mitoxantrone, Estramustine, Carboplatin. The drugs are through IV route, for a certain period followed by a certain period of rest. The drugs usually target the proliferating cells, cancer cells, however, also affect the normal body cells which is the prime side effect of chemotherapy (Nader et al., 2018).

2.12 Staging and Grading in Prostate Cancer

The Gleason grading system is widely used in prognosis of the prostate cancer by assessing the biopsy tissues of the prostate. A Gleason score is given to the prostate tissue when analyzed under the microscope, tissues assigned a higher Gleason Score are more aggressive and have worse prognosis. A total Gleason score out of 10 is assigned to each tissue depending on the histopathological analysis of the tissue. Two scores are assigned out of 5 which are then summed up, the initial score is of the major dominant region and common cell morphology of the prostate tissue, and the second score is assigned to the non-dominant cell pattern (Borley & Feneley, 2009).

2.12.1 Histological Patterns in Prostate Tissue

The pathologist examines the prostate tissue and observes the histopathological patterns according to which the Gleason score is assigned (Gleason, 1992).

Pattern-01: Cancerous cells resemble the normal cells of the prostate tissue, with well-formed glands, and thus the pattern is regarded as differentiated carcinoma.

Pattern-02: In this pattern stroma is observed to be significantly increased, well-formed glands with greater ratio of tissue between them, and is corresponds as moderately differentiated carcinoma.

Pattern-03: the cells in this pattern appear to be darker and begin to leave the glands and invade the surrounding tissues. However, glands in this pattern are still observed as well-formed glands, this pattern also corresponded to moderately differentiated carcinoma.

Patterm-04: Glands are rarely differentiated and recognizable, the cells begin to invade the space around the tissue in an infiltrative pattern, forming neoplastic clumps. This pattern is regarded as poorly differentiated carcinoma.

Pattern-05: Significant overgrowth of cells in the surrounding space is observed regarded as sheets of cells and the tissue has rarely few to no well-differentiated glands. This pattern corresponds to anaplastic carcinoma.

Patterns 1 and 2 are rarely observed, however pattern 03 is commonly observed in the patients of prostate cancer. Patterns 4 and 5 are seen in advanced stages and aggressive cases of prostate cancer.

2.12.2 Gleason Scores and Prognoses

The grading and staging of prostate cancer are based on the Gleason scoring and grading system. The system is based on assigning two scores, the first score is given to the most dominant pattern, the primary score (out of five) where 1 is (the least aggressive) and 5 is (the most aggressive). A secondary score is assigned according to the most common pattern which is also out of 5. The pathologist designates the two grades by looking at the architecture of the prostate gland. At the end, both the scores are summed up and a total score out of 10 is allotted to each patient. The Gleason grading system is based on the potential risk and aggressiveness of the tumor/cancer. The Gleason score range from 2 to 10, where 2 is considered as the most well-differentiated tumor and 10 is the least differentiated and the most aggressive tumor (Cheng et al., 2012). The *Table 2* below represents the grading system of Gleason's score in prostate cancer.

Table 2: Gleason Score and Grade Group

Risk	**Grade Group**	**Gleason Score**
Low	Grade Group 01	6
Intermediate	Grade Group 02	(3+4) =7
	Grade Group 03	(3+4) =7
	Grade Group 04	8
High	Grade Group 05	9-10

2.12.3 Grading Mechanism:

There are five grades in the grading of prostate cancer, that are based on the architectural patterns along with the cytological characteristics of the tissue. The lower the grade number the more differentiated is the tissue. As the grade number increases, the tissue is poorly differentiated. The grades and scores are designated

after observing the architectural patterns and cytological changes between a normal prostate tissue and cancerous tissue. Most observed change in the prostate cancer tissue is the loss of basal layer in the case of prostatic adenocarcinoma.

2.12.4 Gleason Scores Description

Under the system of the Gleason scoring score 02 is assigned to the most well differentiated tumor and 10 is assigned to the least differentiated tumor. The scores are often grouped together according to the similarity in their architectural patterns. Scores 2-4 are considered as well differentiated, score 5-6 are intermediately differentiated, score 07 is regarded as moderate to poor differentiation, and score 8-10 are corresponded as least differentiated and high-grade tumors (Borley & Feneley, 2009).

Gleason Score 01:

Single separate nodules, densely packed, with no interruption and invasion into the surrounding tissue. The glands maintain their normal morphology oval shape, overall, a well differentiated pattern is observed.

Gleason Score 02:

Distinct separate glands are observed with well-formed circumscribed nodules. However, a slight loose pattern is observed in the morphology of the glands, accompanied by minimal neoplastic invasion of the glands in the surrounding tissues, however glands maintain their healthy shape and morphology.

Gleason Score 03:

The most distinctive characteristic of score 03 is the neoplastic invasion of the glands into the surrounding tissue and stroma, the glands become small and microscopic with altered shape and morphology.

Gleason Score 04:

The glands no longer appear to be single and distinct rather have formed fused nets and chains with altered size and shape. Because the glands appear to be fused it gets difficult

to recognize them which is also marked with rare lumen formation and cribriform is observed frequently. The fused glands appear to be scalloped at their edges.

Gleason Score 05:

No glandular differentiation is observed, sheets, cords and nets of cell proliferation are observed. Glands have completely lost their normal morphology, with no luminal spaces.

Chapter 3

Material and Methods

2.13 Ethical Approval

The research project was reviewed and evaluated by the Institutional Review Board (IRB) (IRB No. 04-2021-02/14) of Atta-Ur-Rahman School of Applied Biosciences, National University of Sciences and Technology (NUST), Islamabad. All the experiments of the project were conducted in the Molecular Virology and Viral Oncology Lab. Standard protocols, following the declaration of Helsinki, were used (Association, 2013). In addition to this subjects, intellectually sound individuals provided informed consent to be the part of study after understanding the aim of the project.

3.2 Materials Used

The following tables 1,2,3,4 represent the detailed information of chemicals, enzymes, primers, kits, plasticware, and equipment used in the research respectively.

Table 3: List of Chemicals

Chemicals	**Company**	**Catalog No.**
Absolute ethanol	Sigma-Aldrich	32221-M-2.5L
Agarose	Sigma-Aldrich	N/A
Chloroform	Sigma	UN1888
Distilled water	N/A	N/A
Ethylenediaminetetraacetic acid (EDTA)	Sigma	27285-500G-R
Ethidium bromide	Sigma	UN2810
Ethanol	Sigma	UN170
Isoamyl alcohol	Sigma	59090
Isopropanol	Sigma	UN1219
Magnesium chloride ($MgCl_2$)	Sigma	S290501

Sodium chloride (NaCl)	Sigma	S98881KG
Nuclease-free water	Invitrogen	AM9932
Phenol	Sigma-Aldrich	UN1671
Proteinase K	Solar Bio	P1120
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	436143
Sodium acetate	Sigma	S76701KG
Tris Base	Sigma-Aldrich	93363-500G
Tris-HCl	Sigma-Aldrich	T3253-500G
Triton X	Sigma-Aldrich	UN-3082
Xylene	Sigma	UN1307

Table 4: List of Enzymes, Primers and Kits

Products	**Company**	**Catalog No.**
DNA ladder 100 bp ready to load	Solis BioDyne	07-11-1950
DNA loading dye (6x)	Thermo Fisher	R0611
Primers (β-globin)	Humanizing Genomics Macrogen	N/A
Primers (GP5+/GP6+)	Humanizing Genomics Macrogen	N/A
Primers (HPV-16,18, 31 33, 45, 52, 58)	Humanizing Genomics Macrogen	N/A

Table 5: List of Plasticware

Plasticware	**Company/ Manufacturer**
Aluminum foil	N/A
Centrifuge tubes (15ml)	Bio-fil
Centrifuge tubes (50ml)	Accumax
Disposable syringes	Uniject
EDTA blood vials	LABOVAC

Eppendorf storage box	N/A
Falcon tube racks (15ml)	N/A
Falcon tube racks (50ml)	N/A
Glass measuring cylinder (100ml)	Pyrex
Glass measuring cylinder(250ml)	Pyrex
Gloves	SRITRANG
Ice box	N/A
Microcentrifuge tubes (1.5ml)	FineBiotech UK
Microcentrifuge tubes (2ml)	FineBiotech UK
Multi-tube racks	N/A
PCR tubes	Orange Scientific
PCR tubes (0.2ml)	Fine Bio
PCR tube racks	N/A
Plastic measuring cylinder (500ml)	N/A
Plastic measuring cylinder (1L)	N/A
Pipette tips (0.1-10µl)	Porlab Scientific
Pipette tips (10-100µl)	Porlab Scientific
Pipette tips (100-1000µl)	Porlab Scientific
Reagent bottle (250ml)	Pyrex/SIMAX
Reagent bottle (500ml)	Pyrex/SIMAX
Reagent bottle(1L)	Pyrex/SIMAX

Table 6: List of Equipment

Equipment	**Company**	**Model/Serial No.**
Centrifuge machine	Sigma	136013
Deep freezer (-20ºC)	Haier	bs0190b7x00bhbia0031
Digital weight balance	Shimadzu/Uni Bloc	D450013457S
Electrophoresis power supply	Wealtec	E5W0868
Gel doc system	Cleaver Scientific-UK	130925001

Incubator	Memmert	E-410-1390
Laminar flow cabinet	ESCO	94155
Microcentrifuge	Wealtec	ECW09045386
Microwave oven	Kentax	KNM-20/BW
NanoDrop spectrophotometer	Berthold	84030
pH meter	WTW	1143204
Pipette (0.1-2µl)	Nichipet EX II	H13Z06341
Pipette (2-20µl)	Nichipet EX II	H13Z00661
Pipette (10-100µl)	Nichipet EX II	H13Z08311
Pipette (20-200µl)	Nichipet EX II	J15107741
Pipette (100-1000µl)	Nichipet EX II	H13Z19071
Probe sonicator	Hielscher	UP400S
qPCR	Systaaq AB QuantGene	SYS22RH21100145
Refrigerated centrifuge machine	Hermle (Germany)	66170035
Rotatory microtome	Slee Mainz	Cut6062
Shaking incubator	K&K Scientific Supplier	K-SI 100R
UV transilluminator	Wealtec	ubw250551
Veriti thermal cycler	Applied Bio System	4375786
Vortex	Velp	F202A0173

3.3 Sample Size Calculation:

To find out the sample size of prostate cancer patients for the study, the sample size was calculated using the below-mentioned formula:

$$\boldsymbol{n = \frac{Z^2 P(1-P)}{d^2}}$$

Where,

n = Sample size

Z = Level of confidence (95%)

P = Expected prevalence (2.4%) (Ferlay J, 2024)

d = Precision (5%)

$$\mathbf{n = \frac{Z^2\ P\ (1 - P)}{d^2} = \frac{(1.96)^2\ 0.024\ (1 - 0.024)}{0.05^2} = 35}$$

3.4 Study Population

The research project aims to find the viral suspicion of prostate cancer thus the prevalence of prostate cancer in the Pakistani population was estimated. The study population of the project involved the patients who have been diagnosed with prostate cancer, as their primary cancer and do not have any other secondary malignancy. This was made possible by collaborating with medical professionals of Northwest School of Medicine (NWSM), Peshawar.

3.5 Inclusion and Exclusion Criteria

Inclusion and Exclusion criteria were decided beforehand to ensure that the subjects of the study and their information are not biased and do not interfere with the original results of the study. The study comprised of the patients who had prostate cancer as their primary cancer, with the help of their evaluated histopathology reports. It was also ensured that the study population has not been diagnosed with any other secondary malignancy that alters their health status and later on amends the findings of the study.

3.6 Sampling

A total of 50 Formalin Fixed Paraffin Embedded Tissue (FFPE) samples were collected of prostate tissues. In addition to this demographic data as well histopathological data of the patients were also obtained. The demographic data included some basic life-related questions of the patient including age, residence, dietary habits, lifestyle, family history (of cancers), co-morbidities related to Prostate cancer, and tobacco/ drug usage. This was done to find out any association between these factors and Prostate cancer. Only the tissue samples that had confirmed diagnosis of prostate cancer by their histopathology reports

were further processed. Correct and proper labeling of tissue samples was ensured at the time of sampling.

FFPE samples were collected from the Department of Pathology, Northwest School of Medicine (NWSM), Peshawar, Pakistan.

3.7 H&E and IHC Staining:

Formalin-fixed paraffin-embedded (FFPE) tissue biopsies were processed for hematoxylin and eosin (H&E), and immunohistochemistry (IHC) staining. Tissue processing and staining were conducted by Northwest General Hospital and Research Centre (NWGH & RC), Peshawar, Pakistan. Tissue processing was performed using the standard protocol in a Shandon Citadel 200 (Thermo Scientific). IHC was done using Dako FLEX detection system for primary antibodies, and secondary horseradish peroxidase (HRP) antibody (Suvarna et al., 2018). WHO/ISUP morphological grades were established by trained histopathologists at the institution.

3.8 DNA Extraction:

1. Microtome (Slee Mainz/ Cut6062) was used to cut the FFPE tissue samples into 5-10 um thick sections that were placed in in a 2 mL micro-centrifuge tube.
2. After this, a three-day protocol for DNA extraction was followed that was previously explained by (Pikor et al., 2011) 1 ml of 100% xylene was added to each microcentrifuge tube and the tubes were placed in a shaking water bath at 65°C for 30 minutes to deparaffinize the tissue.
3. After completion of 30 minutes of incubation, the samples were centrifuged at 14000 rpm for 10 minutes which was followed by decanting the supernatant.
4. The above-mentioned two steps were repeated 4-5 times to ensure complete deparaffinization of the tissue sample.
5. It was followed by washing the samples by decreasing the concentration of ethanol to 100%, 80%, and 50% and centrifugation of the sample after each wash at 14000 rpm for 2 minutes, 3 minutes, and 5 minutes respectively.

6. The tissues were then air dried for 15-20 minutes and then 500 µL digestion buffer was added along with 10µL proteinase K. The contents of the digestion buffer are mentioned in the *Table 7*.

Table 7: Contents of Digestion Buffer

Sr. No.	**Tissue Digestion/Lysis Buffer**	**Quantity (500 mL)**
1.	1M Tris-HCl (pH 8.0)	20 mL
2.	0.5M EDTA (pH 8.0)	1 mL
3.	10% SDS	50 mL
4.	Distilled water	404 mL

7. Lastly, the samples were incubated overnight at 50°C.
8. On the following day, the sample tubes were placed in a water bath at 95°C for 10 minutes to inactivate proteinase K.
9. 120µL of NaCl (6M) was added to each tube, which was followed by centrifugation at 14000rpm for 10 minutes to pellet down proteins.
10. This was followed by the transferring of the supernatant to a new 1.5 mL micro-centrifuge tube and 55uL 3M sodium acetate was added followed by vigorous vortex mixing.
11. After this 600µL chilled isopropyl alcohol (IPA) was added and the tubes were incubated at -20°C overnight.
12. On the following day, the IPA-suspended DNA tubes were centrifuged at 14000rpm for 20 minutes at -4°C followed by discarding the supernatant.
13. The pellet was washed with 70% ethanol and centrifuged at 13000rpm for 7 minutes.
14. Lastly, the supernatant was discarded, the pellet was air dried, and later on the DNA was suspended in 50µL of nuclease-free water and stored at -20°C.

3.9 Quantitative Analysis of Extracted DNA

For quantitative analysis of extracted DNA, NanoDrop Spectrophotometer (Berthold/84030) was used, which revealed the concentration as well as purity of the extracted DNA sample. To do so the absorbance of DNA at 260 nm and its 260/280 nm absorbance ratio was obtained. Two to three readings for each sample were taken and an average was taken. The samples having ~1.8, A260/280 ratio and 200-300 ng/uL concentration were then subjected to further downstream processing.

3.10 Qualitative Analysis of Extracted DNA

To assess the quality of the extracted DNA, 1% agarose gel was prepared by adding 1g of agarose in 100 mL of 1X Tris-acetate-EDTA (TAE buffer) and heating it in a microwave oven. The solution was then cooled down and then 5uL of the intercalating dye Ethidium bromide was added to the gel and was thoroughly mixed, and the gel was poured into the casting apparatus and was allowed to solidify. 8 uL of the DNA sample was mixed with 1 uL of loading dye and was loaded into the gel. Gel was run in 1X TAE Buffer at 80 volts, for 15-20 minutes and the bands were visualized using a UV transilluminator. *Table 8* represents 50X TAE buffer recipe and its dilution to 1X TAE buffer.

Table 8: Contents of TAE Buffer

Sr. No.	**TAE Buffer (50X)**	**Quantity (1L)**
1	Tris Base	242 g
2	Glacial Acetic Acid	57.1 mL
3	0.5M EDTA Solution	100 mL
For 1X TAE Buffer Distilled water and 50X TAE Buffer were added in 49:1.		

3.11 Validation of Housekeeping Gene

The presence of extracted DNA from the samples was then further assessed by amplifying the housekeeping gene β-globin. This was done by conducting a conventional PCR using a specific primer for the housekeeping gene. The *Table 9* presents the details for the primer.

Table 9: Details of Housekeeping Gene Primer

Primers	**Sequence 5'-3'**		**Annealing Temp (℃)**	**Amplicon Size (bp)**
β-globin	F	ACACAACTGTGTTCACTAGC	57	110 bp
	R	CAACTTCATCCACGTTCACC		

20 µL PCR reaction mixture was prepared, in which 10.8 µL of nuclease-free water was added, 2.5 µL of buffer was added, 2.0 µL of $MgCl_2$ was added. 1 µL of 10 pM forward primer and 1µL of 10 pM reverse primer (Eurofins | Genomics) was added, 0.2 µL of DNA polymerase, 0.5 µL of dNTP's New England Biolabs was added, and lastly 2 µL of DNA template was added. It was ensured that the PCR mixture was prepared on ice and after the preparation, the reaction mixture was short spin in a microcentrifuge to ensure thorough mixing of all the components.

PCR was carried out on a Veriti Thermal Cycler (Applied BioSystem/4375786) by setting the following PCR profile for β-globin: initial denaturation: 95°C for 5 minutes, denaturation: 95°C for 30 seconds, annealing: 57°C for 30 seconds, and extension: 72°C for 30 seconds repeated for 35-40 cycles, final extension: 72°C for 7 minutes; hold 4°C. After completion of the PCR, the PCR products were analyzed on 2% agarose gel.

For this purpose, the gel was prepared by the previously explained method in section 3.10. In addition to this 3 µL of 100 bp DNA ladder was also loaded in a separate well. The gel was run in 1X TAE buffer for 45 minutes and 80 Volts after which the gel was viewed under the UV transilluminator where the presence of bands pf 110 bp was analyzed to check the validation of extracted genomic DNA in samples.

3.12 Plasmid Designing

For the positive control, in HPV detection, a plasmid was designed, L1 conserved region of HPV. For this purpose, the sequence of L1 gene conserved region was obtained from Pave database, which was followed by an insilico PCR, with GP5+/6+ primers for L1 gene using Snap gene. After the successful amplification with the primers, these gene sequences were synthesized by GBI. Plasmid designing was later followed by NCBI blast and

sequence alignment with the HPV genome sequence using MEGAX software was done to ensure the sequences. The *Table 10* presents the details of the designed plasmid.

Table 10: Details of Plasmid

HPV Region	**Sequence**	**Length (bp)**	**Plasmid**
L1	ATAAGGCACAGGGTCATAACAATGGTGTTTGCT GGCATAATCAATTATTTGTTACTGTGGTAGATACC ACTCGCAGTACCAATTTAACAATATGTGCTTCTA CACAGTCTCCTGTACCTGGGCAATATGATGCTAC CAAATTTAAGCAGTATAGCAGACATGTTGAGGA ATATGATTTGCAGTTTATTTTTCAGTTGTGTACTA TTACTTTAACTGCAGATGTTATGTCCTATATTCAT AGTATGAATAGCAGTATTTTAGAGGATTGGAACT TTGGTGTTCCCCCCCCGCCAACTACTAGTTTGGT GGATACATATCGTTTTGTACAATCTGTTGCTATTA CCTGTCAAAAGGATGCTGCACCGGCTGAAAATA AGGATCCCTATGATAAGTTAAAGTTTTGGAATGT GGATTTAAAGGAAAAGTTTTCTTTAGACTTAGAT CAATATCCCCTTGGACGTAAATTTT	468 bp	pMV

3.13 HPV Detection

The extracted genomes from the prostate tissue that were amplified on housekeeping gene PCR and had satisfactory results of DNA quantification and quality check were then processed for HPV detection. The DNA samples were evaluated for the presence of HPV using the consensus primers GP5+/6+. The

Table **11** presents the details for the GP5+/6+ detection primer along with its annealing temperatures.

Table 11: Details of HPV Detection Primer

Primers	Sequence **5'-3'**		**Target Gene**	Annealing Temp (°C)	Amplicon Size (bp)
GP5+/GP6+	F	TTTGTTACTGTGGTAGATAC	L1	42	150 bp
	R	GAAAAATAAACTGTAAATCA			

Note: Standard codes for wobble are:

M = A / C, R = A / G, W = A / T, Y = C / T

20 µL PCR reaction mixture was prepared, in which 10.8 µL of nuclease-free water was added, 2.5 µL of buffer was added, 2.0 µL of $MgCl_2$ was added. 1µL of 10 pM forward primer and 1µL of 10 pM reverse primer (Eurofins | Genomics) was added, 0.2 1µL of DNA polymerase, 0.5 µL of dNTP's New England Biolabs was added, and lastly 2 µL of DNA template was added. It was ensured that the PCR mixture was prepared on ice and after the preparation, the reaction mixture was short spin in a microcentrifuge to ensure thorough mixing of all the components.

PCR was carried out on a Veriti Thermal Cycler (Applied BioSystem/4375786) by setting the following PCR profile for GP5+/GP6+: 95°C for 5 minutes; denaturation: 94°C for 30 seconds; annealing 42°C for 45 seconds, extension: 72°C for 40 seconds; repeated steps 2-4 for 40 cycles, final extension: 72°C for 7 minutes; hold 4°C.

PCR products were analyzed on 2% agarose gel. For this purpose, the gel was prepared by the previously explained method in section 3.10. In addition to this 3 µL of 100 bp DNA ladder was also loaded in a separate well. The gel was run in 1X TAE buffer for 45 minutes and 80 Volts after which the gel was viewed under the UV transilluminator where the presence of bands of size 150 bp was analyzed to detect the HPV in extracted genomic DNA samples.

Chapter 4

Results

4.1 Clinicopathological Characteristics of Prostate Cancer Patients

The age range for prostate cancer patients in Pakistan under this study was 45-90 years and above, giving an average age of 65. 14% of cases were in the age group 45-60 years, 60% of cases were in the age group 61-75 years, 24% cases were in age group 76-90 years and 2% of cases were 90 above. All the subjects of the study were married individuals. In addition to this, the lifestyle of the patients was also assessed where it was categorized based on their physical activity. Physical activity was characterized as active, moderate, or less active based on its intensity and duration. For an active lifestyle: extensive physical activity for 3 days or moderate physical activity for 5 days per week, for a moderately active lifestyle: moderate physical activity or walking for 3 days per week, and for a less active lifestyle: those who did not fall in above 2 categories was considered.

52% (26/50) of the patients reported active lifestyles, 44% (21/50) of the patients reported moderately active lifestyles, and 4% (2/50) patients reported less active lifestyles.

Tobacco consumption of the patients was also considered among the baseline characteristics of the prostate cancer patients. Surprisingly only 26% (13/50) of the patients were tobacco consumers, while 74% (37/50) were tobacco abstainers.

Furthermore, the comorbidities of subjects under study were also considered, 78% of the individuals reported lower urinary tract symptoms (LUTS) which was the most common problem in the patients of prostate cancer under the study. Following LUTS, Protanomaly/Benign Prostatic Hyperplasia (BPH) was observed in 24% (12/50) patients. Hypertension was observed in 22% (11/50), diabetes was observed in 12% (06/50), and heart disease was observed in 10% (05/50) patients of prostate cancer. *Figure* **4** presents the details of clinicopathological features of prostate cancer patients from Pakistan.

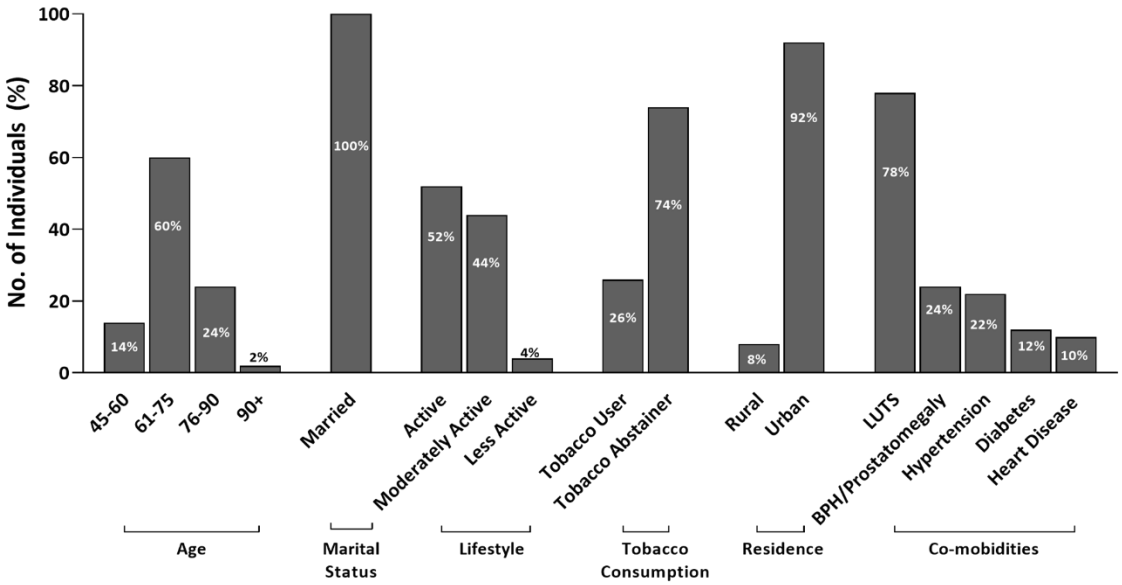


Figure 4: Clinicopathological features of prostate cancer patients of Pakistan. n=50

The *Table 12* presents the details of the clinicopathological features of the Prostate cancer patients.

Table 12: Clinicopathological Features of Prostate Cancer Patients

Clinical Characteristics		**Prostate Cancer Patients (n=50)**
Age (years)	45-60 [a]	07
	61-75	30
	76-90	12
	90 above	01
Marital Status	Married	50
	Unmarried	00
Lifestyle	Active [b]	26
	Moderate Active [c]	21

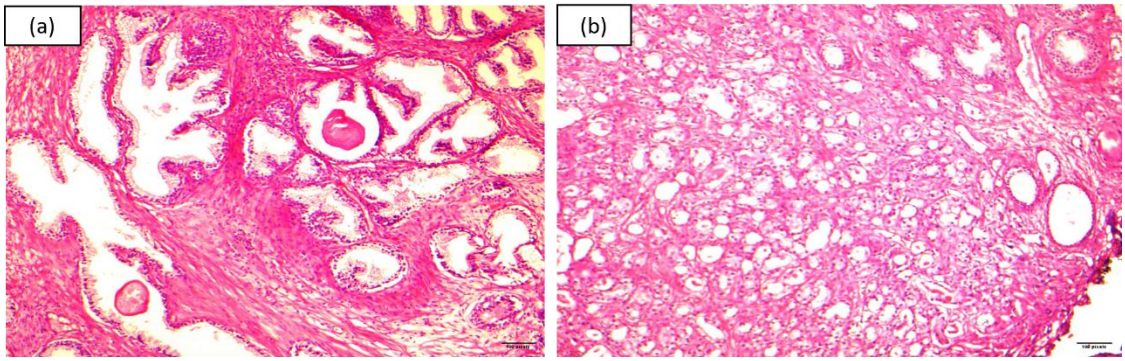
	Less Active [d]	02
Tobacco [e]	Tobacco Users	13
	Tobacco Abstainers	37
Co-morbidities [f]	BPH / Prostatomegaly	12
	LUTS	39
	Diabetes	06
	Hypertension	11
	Heart Diseases	05
Histological Type	Prostatic Acinar Adenocarcinoma	50
Gleason Score	6	11
	7	21
	8	08
	9	09
	10	01

a There was no prostate cancer patient below 45 years of age.
b Active lifestyle: extensive activity for 3 days or moderate physical activity for 5 days per week.
c Moderate active lifestyle: moderate physical activity or walking for 3 days per week.
d Less active lifestyle: those who did not fall into the above 2 categories.
e Cigarettes, cigars, vape, dry tobacco (naswar), etc.
f Pre-existing medical conditions, from the time of cancer diagnosis.

4.2 H&E Staining and IHC Staining

H&E and Immunohistochemistry (IHC) staining revealed the cancer grades for prostate cancer. For comparison normal prostate tissue was assessed along with prostate cancer tissue. The Figure 5 presents microscopic analysis of H&E staining of normal prostate tissue reveals the intact glandular structure of the tissue and uniform lining of the epithelial cells. The stroma around the tissue appears uniform and tissue architecture is observed in an ordered fashion. However, H&E staining of a cancerous prostate tissue reveals disrupted glandular architecture, cellular proliferation, and glands appear to be of irregular size and shape under the microscope. The epithelial lining of the tissue appears to have characteristic cellular and nuclear atypia. In addition to these stromal changes are also seen where inflammation and fibrosis are observed and are the typical characteristic of tumor microenvironment.

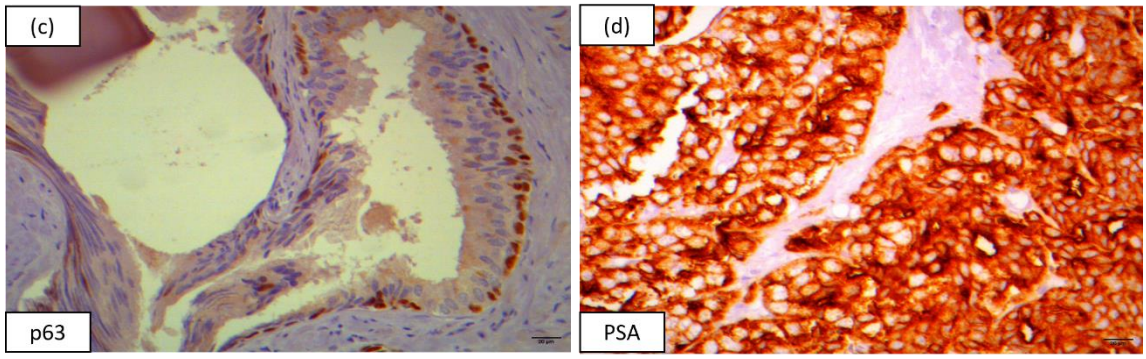
IHC staining of normal prostate tissue shows the intact basal cells of the prostate gland and the nuclear staining with p63 distinguishes basal cells from luminal cells. The overall architecture of the tissue appears to be orderly revealing normal cellular composition. In addition to this IHC of cancerous prostate tissue was performed with PSA to find the level of expression of PSA protein in tissue. PSA staining of a cancerous tissue shows cytoplasmic positivity indicating that cancer cells are expressing the PSA protein. In addition to this, non-neoplastic cell do not show expression for PSA protein thus this helps in distinguishing cancerous cells from normal prostate cells. Figure 6 presents IHC of a normal prostate tissue and prostatic adenocarcinoma tissue.



Normal Prostate Tissue, H&E Staining: Well-formed regular glands.

Prostate Cancer Tissue, H&E Staining: Irregular glandular structure, nuclear atypia.

Figure 5: H&E Staining of normal and prostate cancer tissue; Magnification 400X; Scale 20um



Normal Prostate Tissue, IHC Staining: Strong membranous expression of p63 in normal prostate tissue.

Prostatic Acinar Adenocarcinoma, IHC Staining: Strong and diffused expression of PSA.

Figure 6: IHC Staining of normal and prostate cancer tissue, Magnification 400X, Scale 20um

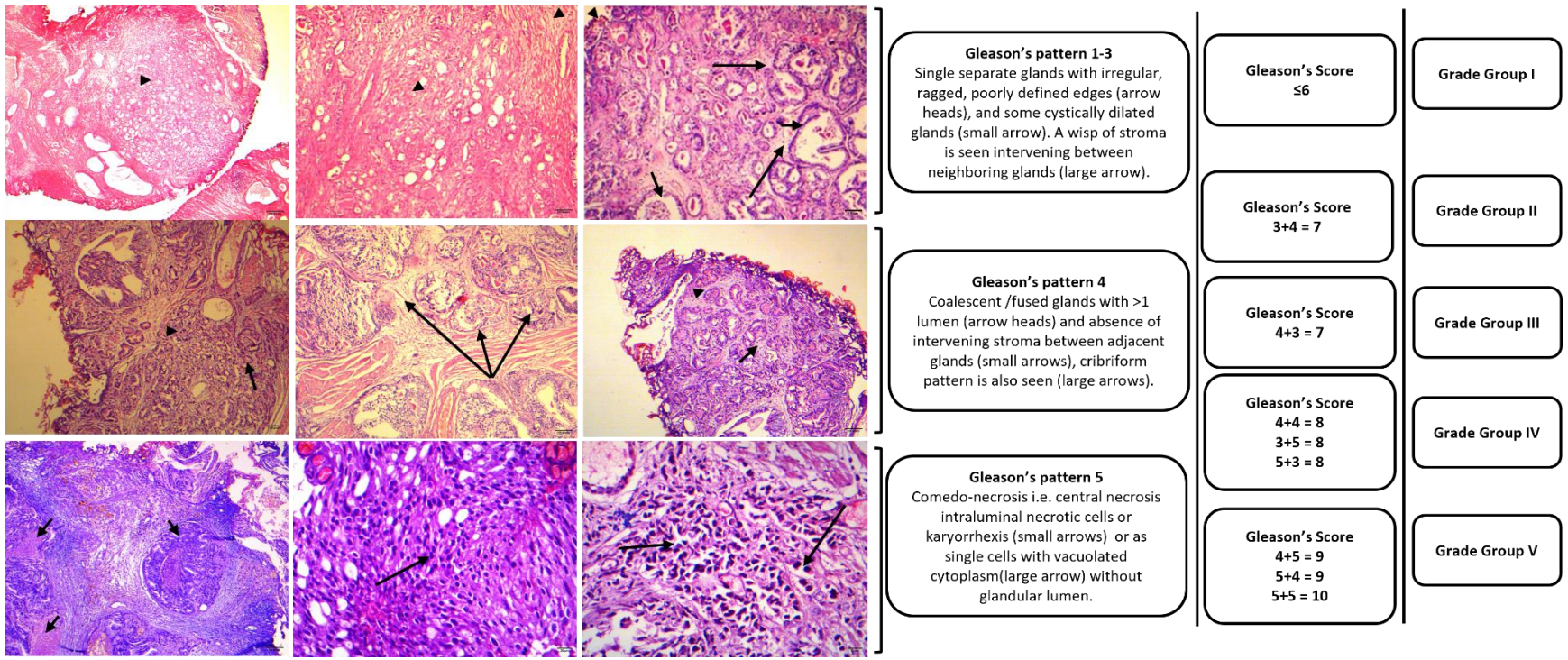


Figure 7: Histological Features of Prostate Tissues, Magnification 400X, Scale 20um

In addition to this the Gleason pattern of the subjects under study were also observed according to which they were assigned their Gleason score. The Figure 7 shows the Gleason patterns that were observed in the patients of the study.

4.3 Histopathological Characteristics of Prostate Cancer Patients

For Histopathological features, 100% (50/50) cases were of prostatic acinar adenocarcinoma. represents the percentage distribution of histological types of prostate cancer cases for the Pakistani population. According to Gleason score and grading of the prostate cancer, 22% (11/50) had score 6/10, 42% (21/50) cases had score 7/10, 16% (8/50) cases had score 8/10, 18% (9/50) cases had score 9/10 and 2% cases (01/50) had score 10/10 presented. 64% (32/50) patients had low to moderate risk cancers and were among grade group I, II, III and 36% (18/50) had high risk cancers and were among grade groups IV and V. Figure 8 illustrates graphical representation of cancer types, Gleason scores and grade groups of patients.

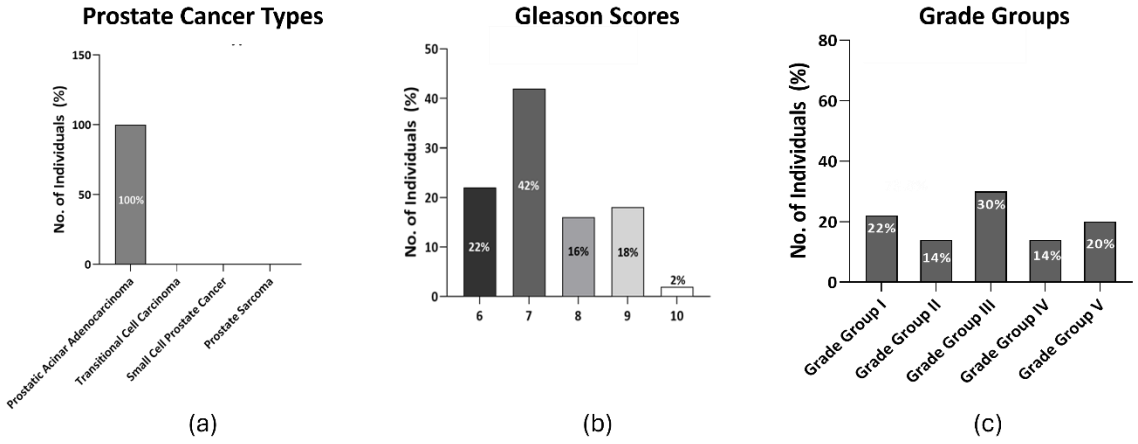


Figure 8: (a) Types of prostate cancer in patients (b) Gleason Scores of prostate cancer patients (c) Distribution of grade groups of prostate cancer patients

4.4 Validation of Housekeeping Gene

β-Globin was used as a housekeeping gene to check the integrity of the extracted DNA. 110 bp bands were observed on a 2% agarose gel of the samples who had their DNA amplified against β-Globin. The Figure 9 represents the amplification of extracted DNA from prostate cancer tissues.

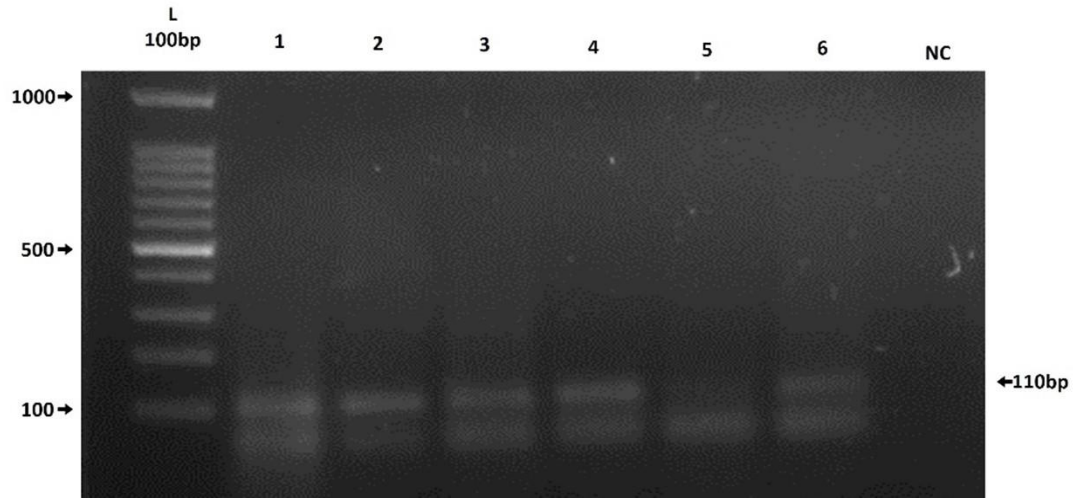


Figure 9: DNA confirmation of prostate cancer patients. PCR-based amplification of 110bp long region of housekeeping gene, β-Globin. L represents the 100bp ladder, and lanes 1 to 7 are β-Globin amplified samples. 2% agarose gel was used

4.5 HPV Detection:

The nucleic acid extracted from the prostate cancer biopsies was then processed to detect the presence of HPV. For HPV detection GP5+/GP6+ (150bp) consensus primers were used. However, no samples were amplified, and HPV was detected in 0% (0/50) samples of prostate biopsies. The Figure 10 shows none of the DNA was amplified with GP5+/GP6+, thus no HPV was detected.

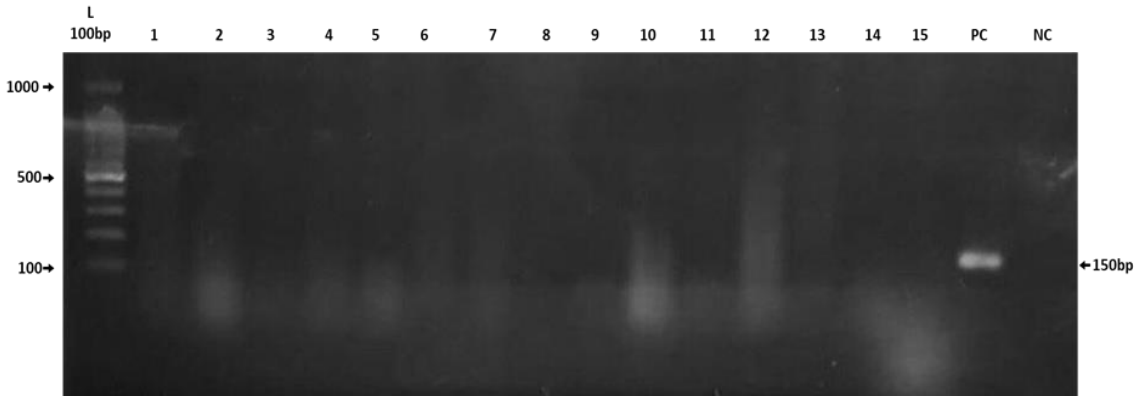


Figure 10: HPV detection in prostate cancer through PCR-based amplification of 150bp long GP5+/6+ consensus region, Lanes 1 to 15 show HPV-negative prostate cancer samples. L represents the 100bp ladder, PC is positive control and NC is negative control. 2% agarose gel was used.

Chapter 5

Discussion:

The etiological association between prostate cancer and HPV is quite controversial and results remain elusive, as there are studies that have shown an association between prostate cancer and HPV while there are studies that reveal no correlation and between prostate cancer and HPV infection. This is the first-ever study conducted in Pakistan to find the association between HPV and prostate cancer. This study conducted on prostate cancer patients from Pakistan showed no association between HPV and prostate cancer. HPV was detected in none of 0%, (0/50) prostate cancer biopsies suggesting no significant relation between prostate cancer development and HPV infection. A study reported no association between HPV (16,18 and 31 genotypes) via antibody assessment which was done by ELISA (enzyme-linked immunosorbent assay), as a risk in the development of prostate cancer in older American men (n=616). However slight positive yet non-significant findings were observed for HPV-16 and HPV-31. The results of the study have concluded that HPV does not appear to be associated with the risk of the development of prostate cancer (Sutcliffe et al., 2010).

Another study conducted in 2009, on Brazilian men, concluded their study that despite the high prevalence of HPV in North Brazil, no association between HPV and prostate cancer was found. The study was conducted using a highly sensitive test, linear array HPV genotyping. The study was conducted to detect 37 high and low-risk HPV genotypes where only 3% (2/65) prevalence of HPV was found in prostate cancer samples, the samples had co-infection of HPV-16 and 84. HPV was not detected in any BPH sample under study (Silvestre et al., 2009). A 2012 study conducted on British population with the aim to find role of multiple pathogens in prostate oncogenesis found a 1% prevalence of HPV in 100 prostate biopsies using INNO-LIPA HPV genotyping and PCR, concluding no association of HPV with prostate cancer (Groom et al., 2012). Another study in the same year 2012 from Prague has also reported no association between HPV and prostate cancer. The study

detected the HPV genome in only 2% of 146 prostate biopsies using PCR and ELISA techniques (Tachezy et al., 2012). In addition to this a study from Turkey in 2017 has also reported no significant association between HPV and prostate cancer. The study used qPCR technique for detection purpose on 60 prostate biopsies and detected HPV in 1.6% of the samples (Aydin et al., 2017).

In 2020 a study reported no association between HPV and prostate cancer however HPV can contribute to chronic or recurrent inflammation of the prostate gland. The study was conducted on 58 prostate cancer samples and 32 non-malignant prostate tissues from Iranian population. The findings of the study have shown that the HPV genome was detected in 32.7% (19/58) prostate cancer cases and 15.6% (5/32) control cases. High-risk HPV genotypes, HPV-16 and HPV-18 were found to be more prevalent at 47.4% and 31.6% respectively in the infected prostate cancer tissues. In addition to this, the role of HPV was also assessed in causing inflammation and angiogenesis where it was found that the expression of tumor suppressor (Rb and p53) genes was significantly downregulated in prostate cancer tissues compared to control. However, the levels of anti-apoptotic mediators and those involved in angiogenesis were significantly increased in prostate cancer tissues, compared to HPV-negative and control tissues. The authors have concluded that no statistically significant relation was found between HPV and prostate cancer (Nahand et al., 2020).

Contrarily some studies have reported an association between HPV and prostate cancer across the globe, A study conducted in 2010 inspected the role of various pathogenic organisms including polyomaviruses BKV, JCV, and SV40; human papillomaviruses (HPVs), and human cytomegalovirus (HCMV). This was done by detecting the viral genome in prostate tissues which was followed by Taqman method. The study was conducted on 55 patients of prostate cancer and 75 subjects as controls were part of the study which was conducted on the Mexican population. HPV genome was detected in 20% (11/55) of prostate cancer cases and 5.3% (5/75) of control cases. The findings of the study conclude that there exists a positive relation between HPV infection and the development of prostate cancer and the infection increases the risk of development of prostate cancer 3.98 times (Martinez-Fierro et al., 2010). In addition to this another study conducted in

2013 has also investigated the clinical significance of HPV in prostate cancer development in Italy, by tissue microarray, PCR, sequencing, and IHC. It was found that 74.6% (112/150) of prostate cancer patients had positive expression for HPV E7 protein, revealing a significant positive association between HPV and prostate cancer. This was further confirmed by PCR and Sequencing which confirmed the HPV infection and revealed the presence of HPV-16 in prostate cancer cases (Pascale et al., 2013). India, a neighboring country to Pakistan, reported a 41% HPV prevalence in prostate cancer in a 2015 study involving 150 prostate biopsies using PCR (Singh et al., 2015). In 2018, a study was conducted to evaluate the role of HPV in prostate cancer in the Mexican population. This study has also reported the presence of HPV oncogenic proteins in Mexican men by PCR and viral genotyping. The findings suggested a strong positive association between HPV and prostate cancer as the light microscopy also revealed the presence of koilocytes which are a major characteristic of HPV induced carcinoma. High-risk HPV was detected in 19.6% (37/189) of prostate cancer samples and in 9.6% (16/167) of BPH samples. In addition to this, immunohistochemistry was conducted and overexpression of p16-INK4A was found in HPV-positive samples suggesting the presence of E7 viral oncoprotein (Medel-Flores et al., 2018). A study conducted on British population in 2023 reported a 32.7% prevalence in 49 biopsies using PCR, Sanger sequencing (Ahmed et al., 2023). 2023). A 2023 study from China found a 16.9% HPV prevalence in 59 prostate biopsies using Illumina sequencing, further supporting the involvement of HPV in prostate oncogenesis (Lang et al., 2023).

However, some studies have reported the presence of HPV in the early stages of prostate cancer, where the samples of Benign Prostatic Hyperplasia (BPH) were taken, however, no HPV has been detected in the prostate cancer samples. The study was conducted on 52 Australian men who initially had BPH and subsequently developed prostate cancer. Tissue samples of the same patients were obtained of BPH as well as prostate cancer tissue. The analysis for the detection of HPV was conducted by PCR which was followed by immunohistochemistry staining and followed by RNA- Seq data from The Cancer Genome Atlas (TCGA). The HPV PCR screening was conducted on 28 of 52 sets of BPH and later prostate cancer cases. HPV L1 gene was identified in 46% (13/28) benign samples and 29% (8/28) cases of prostate cancer. In addition to this HPV E7 gene was identified in 82%

(23/28) cases of BPH and 68% (19/28) subsequent prostate cancer cases. HPV genotypes were also identified, HPV-16 was found in 15% of BPH cases and 3% of prostate cancer cases and HPV-18 was found in 26% BPH cases and 16% of prostate cancer cases. The immunohistochemistry analysis revealed the high expression of E7 oncoprotein in 82% (23/28) BPH cases and 29% (8/28) cases of prostate cancer. The difference was found to be statistically significant. However, expression of PSA level was found high in 50% (26/52) of cases of prostate cancer, compared to BPH cases. High-risk HPVs were found to be more prevalent in BPH cases with higher expression of E7 proteins suggesting that HPV oncogenic activity is an initial phenomenon in prostate oncogenesis. In addition to this TCGA RNA-Seq data suggests that HPV was biologically active in prostate tumor samples (Glenn et al., 2017).

Heatmap in **Figure 11** shows the prevalence of HPV in prostate cancer reported in multiple studies from different parts of the world.

Country	Prevalence
Japan	0%
Sweden	
Saudia Arabia	
Netherlands	
Chile	
Brazil	
UK	1%
Turkey	1.7%
Prague	2%

Country	Prevalence
Morocco	16%
Greece	16%
China	17%
USA	20%
Australia	29%

Country	Prevalence
Iran	36%
India	41%
Argentina	42%
France	53%
Canada	56%
Germany	68%
Italy	75%

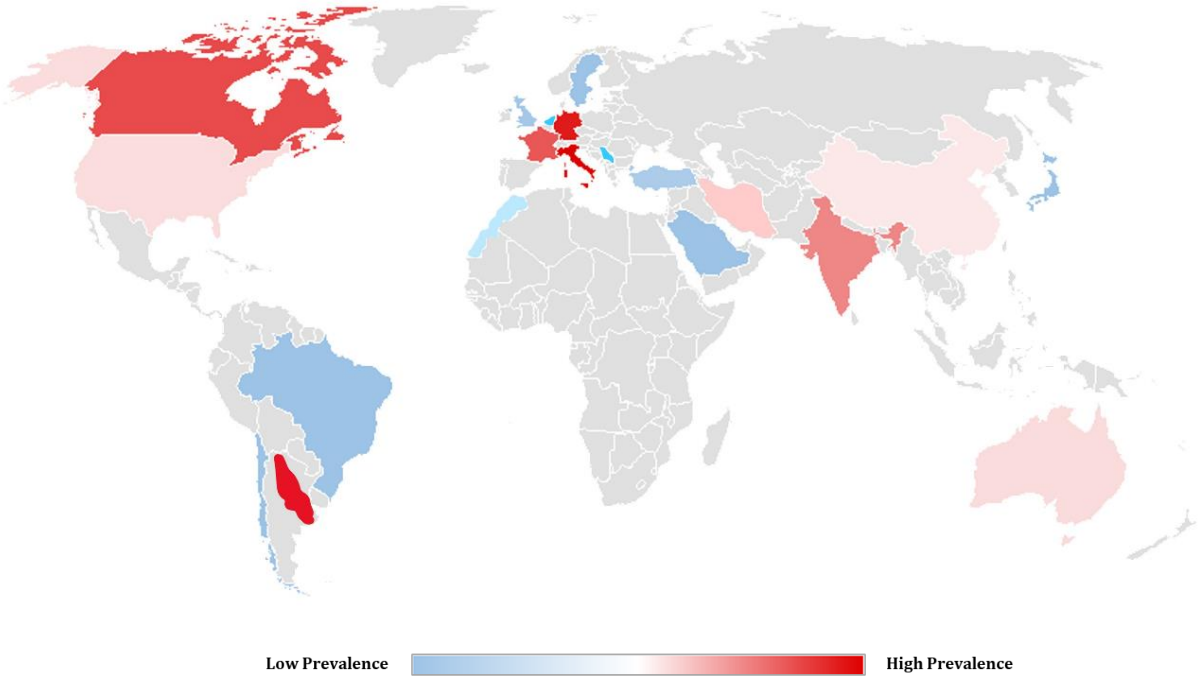


Figure 11: Geographical Heatmap

In this study, prostatic acinar adenocarcinoma was found to be the most prevalent type of prostate cancer in the Pakistani population. 100% of the cases under study were of prostatic acinar adenocarcinoma. In addition, the Gleason Score of the prostate cancer patients was also recorded to assess the prostate gland and abnormalities in its cells and tissues. 22% (11/50) patients had their Gleason Score 6/10, 42% (21/50) of patients had a Gleason Score 7/10, 16% (08/50) of patients had their Gleason Score 8/10, 18% (9/50) patients had their Gleason Score 9/10, and 2% (01/50) had their Gleason Score 10/10. Literature also shows that most of the cases (>95%) cases of prostate cancer are prostatic adenocarcinoma. The findings of the study concord with already reported stats. (Swallow & Kirby, 2008).

Furthermore, other clinical features related to prostate cancer were also evaluated. PSA levels of all patients with prostate cancer were analyzed which was observed significantly high in most cases. 36% (18/50) patients had their PSA level ranging between 0-50ng/mL, while 20% (10/50) had their PSA level between 50-100ng/mL. And 42% (21/50) of patients had their PSA level over 100ng/mL. According to the National Cancer Institute, the rise in PSA increases the risk of development of prostate cancer. The cohort of our study also showed that the people receiving treatment for prostate cancer presented a noteworthy decline in PSA level (Fall et al., 2007).

In the case of prostate cancer, 78% (39/50) of the cases reported lower urinary tract symptoms (LUTS), 24% (12/50) reported benign prostatic hyperplasia (BPH) and Prostatomegaly. Although LUTS has long been linked with the development of prostate cancer, the association has not been proven statistically. BPH is found to be a risk factor in the development of prostate cancer, and BPH can lead to symptoms of the lower urinary tract including urinary problems nocturia, daytime urgency, urinary hesitancy, and weak stream (Nordström et al., 2021).

According to the reports of the American Cancer Society, 60% of men diagnosed with prostate cancer are above the age group of 65 years and is rarely diagnosed in men under the age 40 years (Bechis et al., 2011).

In addition to this, a study conducted on different risk factors of the prostate has also reported that advancing age is a significant risk factor for prostate cancer (Leitzmann & Rohrmann, 2012).

Another study has also reported that most cases of prostate cancer are diagnosed after the age of 65 years (Grönberg, 2003).

The cohort of this study also agrees with the general global trend of age group in prostate cancer. 60% of the subjects in the study were in the age group 61-75 years and 24% were in the age group 76-90%. Moreover, 2% of cases were above 90 years. However, a minor proportion of the subjects of the study 14% were between the age group 45-60 years.

The effects of contemporary lifestyle choices including physical activity levels and tobacco consumption were also assessed which might have played a role in the development of prostate cancer. 52% (26/50) of subjects of the study reported their active lifestyle, 44% (22/50) reported a moderately active lifestyle and 4% (02/50) reported sedentary lifestyle. Contrarily in literature active lifestyle and enhanced physical activity lowers the risk of development of prostate cancer. It has been reported that low physical activity and increased body weight/ obesity contribute to the development of aggressive prostate cancer. Obesity has also been linked to the development of comorbidities and insulin resistance which are also risk factors for prostate cancer (Kenfield et al., 2011).

Furthermore, tobacco consumption is also considered a substantial risk factor for cancer development. A systematic review and meta-analysis conducted by Fahad Islami and colleagues have concluded that there is a significant association between smoking and alcohol usage with development of fatal prostate cancer (Islami et al., 2014).

Another study has also reported that smoking remarkably elevates the risk of prostate cancer (Hsing et al., 1990).

Contrarily the cohort of this study opposes this global trend, as 74% of the prostate cancer patients were tobacco abstainers. This contradiction with the global trend can be justified by the idea that the Pakistani population abstains from alcohol consumption because of cultural and religious implications however smoking is observed in Pakistani men. The present study shows no significant relation between smoking and prostate cancer.

In addition to this, the dietary habits of the subjects of the study were also assessed. A significant proportion of the cohort reported their dietary habits as balanced along with a high intake of green vegetables. However, the literature supports that the dietary pattern

that has high calcium intake increases the risk of developing prostate cancer. A high intake of calcium is known to downregulate the active form of vitamin D in blood circulation, which plays a major role as a tumor suppressor in prostate cancer (Stroomberg et al., 2021).

In addition to this high intake of dietary fat and meat have also been associated with a high risk of development of prostate cancer. The high intake of red meat and fat has been associated with the risk of development of cancer, as Heterocyclic Amines (HCA's) are the potential carcinogens in these foods and get active when cooked at high temperatures (Peisch et al., 2017)

Conclusion and Future Perspectives

The association between prostate cancer and HPV infections remains elusive. This is the first study from Pakistan to explore the etiological association between HPV and Prostate cancer. However, no HPV was detected in Pakistani men with prostate cancer. However, it is important to conduct further studies in future with greater sample size and diverse sampling for better understanding of role of HPV in prostate cancer. In addition to this, other techniques including Immunohistochemistry should also be conducted on the prostate cancer tissues to detect the presence of oncogenic proteins E6 and E7 of HPV in samples. Other more sensitive techniques can also be introduced to check the viral load and biological activity of the virus in cancer tissues for deeper insight in this regard. Pakistan is a developing country therefore HPV poses critical health concerns to the country. It is mandatory for the government to raise awareness among masses regarding HPV infection and provide education on STD's and introduce local screening programs to make effective strategies including vaccination and other treatment options according to Pakistani population

Chapter 6

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Appendix I: IRB Certificate

National University of Sciences & Technology
(NUST) Sector H-12, Islamabad

Research Project Title: Evaluation of therapeutic potential of a natural HDAC inhibitor sulforaphane against HPV infected carcinomas.

Name of Principal Investigator	Dr. Saira Justin
Duration	2.5 Years
Name of School/Department	ASAB -NUST
IRB No.	04-2021-02/14

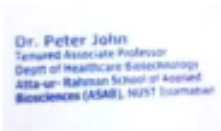
The project entitled above has been reviewed by the NUST Ethical Review Committee Meeting held on **April 16, 2021.** Keeping in view the following mentioned areas.

- Qualification and Expertise of the Principle Investigator.
- Proposed Goals of the Study
- Selection Criteria of the Subjects
- Informed Consent in local language if required
- Potential Problems
- Research Design and Methods
- Potential Benefits of the study
- Risks of the Study
- Assessment & Management of Risk
- Confidentiality & Conflict of Interest.

The Committee approves above entitled project on scale and criteria given below to be implemented before/during project execution.

- Safety measures of carcinogenic chemicals.
- Designated space/work place/rooms for the experimental work.
- Protection of other research students/lab staff/animals from chemical hazardous.

The Ethical Review Committee reserves the rights to re-review the project during the project execution to address the suggested guidelines.



Dr. Peter John

President Ethical Review Committee

Appendix II: Patient Consent Form

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES & TECHNOLOGY
SECTOR H-12, ISLAMABAD.

HEALTHY PERSON INFORMED CONSENT FORM

Research Project, ASAB, NUST — NUST Healthy Person Reference

Participant's Details:

Name:

Age:

Gender:

Blood Group:

Body Mass Index:

Marital status:

Occupation:

Religion:

Hometown:

Current city:

Additional Notes:

Lifestyle:

Health Status:

Do you smoke?

Do you consume alcohol?

Do you use recreational drugs?

Do you have diabetes?

Do you have hypertension?

Do you have any of CVDs?

Are you allergic to anything?

CONSENT FORM

I am willingly volunteering to provide a blood sample for this research. I have been briefed about the research and have been assured that my personal information will be kept confidential & completely anonymous at all times. I am also free to leave this research at any time, at will.

Participant's Signature

Resident Doctor/ Student

میں اس تحقیق کے لیئے رضا کارانہ طور پر اپنے خون ☐ کا نمونہ دینے کیلیئے تیار ہوں۔ مجھے اس تحقیق کے متعلق بنیادی معلومات دی گئی ہیں اور یہ یقین دلایا گیا ہے کہ اس تحقیق کے دوران یا بعد میں میرے ذاتی کوائف صیغہ راز میں رہیں گے۔ مجھے اس تحقیق سے کسی بھی وقت الگ ہونے کی مکمل آزادی ہے۔

Appendix III: Patient Health Assessment Form

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES (ASAB)
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY (NUST

Prostate Cancer Patient Health Assessment Form

Name:	**Hometown:**
Gender:	**Current City/Residence:**
Age:	**Occupation:**
Blood group:	**Marital Status:**
Height: **Weight:**	**Ethnicity/Race:**

Please select all that apply.

1) Lifestyle:
a. Sedentary b. Moderately active c. Active

2) Dietary habits:
a. Balanced b. Red meat c. Oil, Butter and Ghee d. Milk, Yoghurt, Paneer,
e. Green Vegetables like spinach, broccoli

3) Do you smoke?
a. Yes b. No
If yes, please specify: 1. Cigarette 2. Vape 3. Naswar pouches 4: Other

4) Do you consume alcohol or recreational drugs?
a. Yes b. No c. If yes, please specify the type:

5) Have you ever had exposure to chemicals, metal, or radiation?
a. Yes b. No c. If yes, please specify:

6) Have you had any of the following prostate related diseases or conditions?
a. Prostatitis b. Benign Prostatic Hyperplasia c. Trouble in urination
d. Other, please specify:

7) Type of Prostate Cancer:
a. Adenocarcinoma b. Transitional Cell c. Small Cell Prostate (Neuroendocrine)
d. Prostate Sarcoma e. Other
If other, please specify:

8) Cancer Gleason Score: a. 5 b. 6 c. 7 d. 8 e. 9 f. 10

9) PSA (Prostate Specific Antigen) Level: _____

10) Have you received any treatment for Prostate Cancer:
a. Yes b. No
If yes, please specify:

11) Result of the given treatment:
a. Effective b. Not Effective Specify the details:

12) Current condition of the cancer patient:
a. Currently present b. Remission c. Cured

13) Family history of Prostate Cancer or any other cancers:
a. Yes b. No
c. If yes, please specify the relationship and type of cancer:

Appendix III: Patient Health Assessment Form

Description of lifestyle:

Lifestyle

1. **Sedentary Lifestyle:**
Very little to no physical activity, Prolonged sitting, Lack of movement, Health risks like Obesity and Cardiovascular diseases etc.

2. **Moderately Active Lifestyle:**
Occasional physical activities but often have routines that involve limited exercise, such as minimal walking, light household chores, or infrequent workouts.

3. **Active Lifestyle:**
Regular and sufficient physical activity and exercise. Physical fitness, Balanced diet, and Social and psychological well-being.

Dietary habits

1. **Balanced diet:**
Diet contains a mix of different foods in the right amounts to stay healthy. It includes fruits, vegetables, grains, lean proteins, and healthy fats. Drink plenty of water, control portion sizes, and limit added sugars and salt.

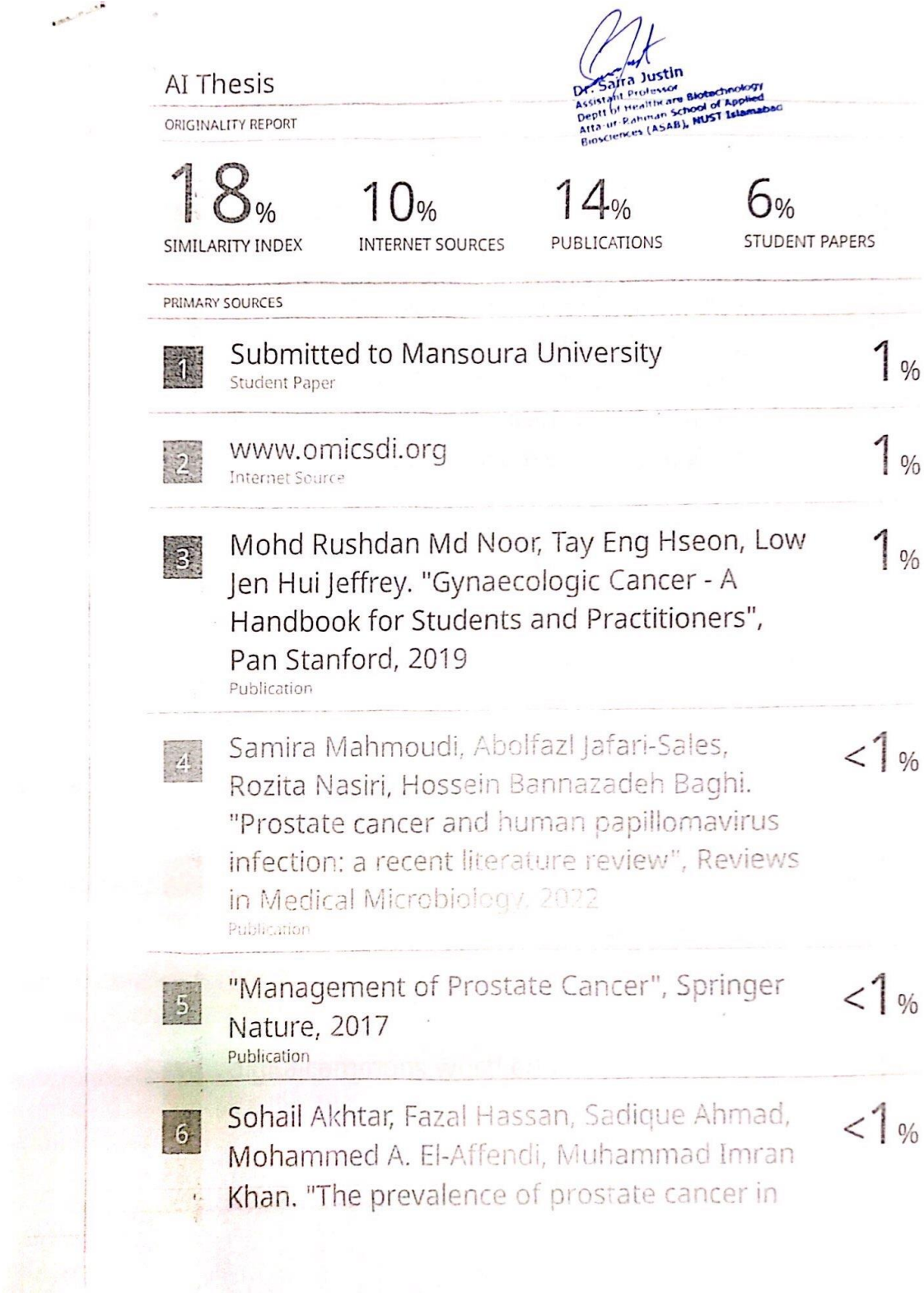
2. **High-protein diet:**
Diet contains a lot of foods rich in protein like meat, eggs, dairy, and beans.

3. **High-fat diet:**
Diet contains a lot of foods with fats like oils, butter, and fatty meats (red meat).

4. **High- calcium diet:**

Cheese, paneer, yogurt, milk, beans, lentils, almonds, green vegetables.

Appendix IV: Plagiarism Report



AI Thesis

ORIGINALITY REPORT

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

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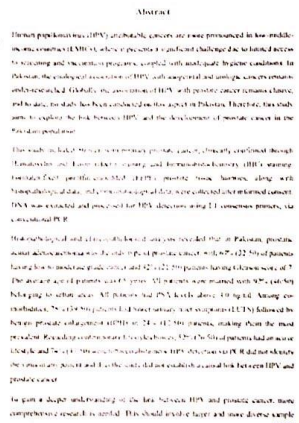

Abstract

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad