Prevalence and Genotype Distribution of Human Papillomavirus (HPV) in Renal Cell Carcinoma Patients in Pakistan

MS Healthcare Biotechnology (2021-23)

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2023

Prevalence and Genotype Distribution of Human Papillomavirus (HPV) in Renal Cell Carcinoma Patients in Pakistan

A thesis submitted in partial fulfilment of the requirement for the degree of Master's of Science in Healthcare Biotechnology

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2023

FORM TH-4

National University of Sciences & Technology

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I certify that this research work entitled "Prevalence and Genotype Distribution of Human Papillomavirus (HPV) in Renal Cell Carcinoma Patients in Pakistan" is my own work. The work has not been presented elsewhere for assessment. The materials that have been used from other sources have been properly acknowledged/referred.

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DEDICATION

With utmost gratitude, my heartfelt dedication goes to Almighty ALLAH for His blessings, followed by my esteemed parents, and beloved siblings, for their unwavering support and encouragement.

ACKNOWLEDGMENTS

In the name of Allah, the Most Gracious and Merciful, I express my profound gratitude to **Allah Almighty** for granting me the capability and privileges to proficiently conclude my dissertation. Firstly, I would like to express my heartfelt appreciation to my supervisor, **Dr. Saira Justin**, whose exceptional guidance, constant support, and patience during this project have been invaluable. I am also really grateful to my GEC members, **Dr. Aneela Javed, Dr. Maria Shabbir,** and **Dr. Naureen Ehsan Illahi**, for their stupendous encouragement and guidance during this period. Additionally, I would like to acknowledge and express my gratitude to **Prof. Dr. Muhammad Asghar**, the Principal of Atta-Ur-Rahman School of Applied Biosciences (ASAB), and **Dr. Sobia Manzoor**, Head of the Department of Healthcare Biotechnology, for providing me a platform to execute my research in a professional and competent environment. Their encouragement and affirmation have been indispensable in assisting me to mold my thesis and ensuring this academic venture possible.

I would like to express my deep gratitude to my polymath labmates for their insightful remarks and contributions during this study, especially to **Muhammad Irfan, Hafsa Yousaf**, and **Ayesha Asim**. I am also deeply grateful to my altruistic friend, **Muhammad Muaz**, for his unwavering moral support and input during this academic period. My sincere gratitude to all of my friends for their steadfast motivation, diligence, and moral encouragement. Last but certainly not least, I would like to extend my sincere gratitude to my beloved parents **Mr. & Mrs. Zulfiqar Ali**, my siblings, **Almas Zulfiqar**, **Farzam Taiyal Zulfiqar**, and **Khuimin Ali**, and all my family members for their everlasting support, prayers, and love. I am truly grateful for their presence in my life.

Sabahat Zulfiqar

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CHAPTER 6

List of Abbreviations

%	Percentage
µl	Micro-liter
µm	Micro-meter
°C	Degree celsius
AJCC	American Joint Committee on Cancer
APOBEC3	Apolipoprotein B mRNA editing enzyme catalytic polypeptide 3
β-actin	Beta-actin
β-globin	Beta-globin
CASP8	Caspase protein 8
ccRCC	Clear cell renal cell carcinoma
CDK2	Cyclin-dependent kinase 2
cIAP-2	Cellular inhibitor of apoptosis protein 2
cGAS–STING	Cyclic GMP-AMP synthase- stimulator of interferon genes
chrRCC	Chromophobe renal cell carcinoma
DNA	Deoxyribonucleic acid
ds- DNA	Double-stranded deoxyribonucleic acid
E6-AP	E6-associated protein
EBV	Epstein-bar virus
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FFPE	Formalin-fixed paraffin-embedded
FGFR2b	Fibroblast growth factor receptor 2b
g	Gram
HGFR	Hepatocyte growth factor receptor
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HPV +ve	Human papillomavirus postive
HPV -ve	Human papillomavirus negative
HR-HPV	High-risk human papillomavirus
IFN-α	Interferon αlpha

IFN-β	Interferon βeta
IFN-κ	Interferon κappa
IPA	Isopropyl alcohol
IRF-3	Interferon regulatory factor 3
JAK	Janus-kinase
kb	Kilobase
kDa	Kilodalton
KGFR	Keratinocyte growth factor receptor
L	Liter
LR-HPV	Low-risk human papillomavirus
M	Molar
MAPK	Mitogen-activated protein kinase
mg	Milli-gram
$MgCl_2$	Magnesium chloride
MHC-class I	Major-histocompatibility complex class I
miRNA	Micro-ribonucleic acid
mTOR	Mammalian target of rapamycin
NA	Not available
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa b
ORF	Open reading frames
ORI	Origin of replication
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PML-NBs	Promyelocytic leukemia nuclear bodies
pRCC	Papillary renal cell carcinoma
Rb	Retinoblastoma protein
RCC	Renal cell carcinoma
rpm	Revolutions per minute
SE	Standard error
SDS	Sodium dodecyl sulfate
SPSS	Statistical package for the social sciences
STAT	Signal transducer and activator of transcription

TAE	Tris-acetate ethylenediaminetetraacetic acid
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TNFR1	Tumor necrosis factor receptor 1
TNM	Tumor-node-metastasis
TLR3	Toll-like receptor-3
TYK2	Tyrosine kinase 2
UBE3A	Ubiquitin-protein ligase E3A
URR	Upstream regulatory region
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-lindau
WHO	World Health Organization
XAF1	XIAP associated factor 1
XRCC1	X-ray repair cross-complementing 1

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ABSTRACT

Human papillomavirus (HPV) is the most common sexually transmitted infection. High-risk HPV (HR-HPV) strains-induced infections are associated with invasive cancer development, especially cervical cancer. HPV infections have a strong correlation with the emergence of other malignancies, including that of the oesophageal, vulva, vagina, penis, or anus. Since HPV-related renal cell malignancy is still controversial possibly due to limited data on the etiological association of HPV with renal cell carcinoma (RCC) or conflicting research results, this study was designed to investigate the prevalence of HPV and the risk of developing RCC with respect to the Pakistani population. The study included 50 healthy individuals and 48 RCC patients. The demographic data of the participants were collected with their informed consent. Blood samples from healthy individuals and tissue samples from RCC patients were collected along with their histopathological reports. DNA was extracted, and HPV testing was performed via conventional PCR.

This study showed that the ubiquity of RCC is significantly greater in males (63%) as compared to females (37%). The age of 44% of patients was below 51 years, 40% of patients were between 51 and 70 years and 16% were between 71-90 years of age. Histologically, clear cell renal cell carcinoma (ccRCC) was the most prevalent type (81%). While papillary renal cell carcinoma (pRCC) and chromophobe renal cell carcinoma (chrRCC) had 7% and 6% of prevalence respectively. At Stages II and III, 38% and 33% of the cases were diagnosed respectively. Whereas, 38% and 31% of the cases were diagnosed at G2 and G1 grades respectively concluding that RCC is diagnosed at early and intermediate stages. Moreover, the prevalence of HPV was observed in 10.4% of the total RCC cases. Further confirmation and establishment of a correlation between HPV and RCC can be attained by increasing the sample

size and the implementation of HPV genotype-specific testing, with particular emphasis on detecting the presence of HR-HPV genotypes, notably HPV-16, & -18.

INTRODUCTION

Human Papillomavirus (HPV)

HPV is a non-enveloped, double-stranded DNA (ds-DNA) virus that belongs to the Papillomaviridae family. HPV is a highly prevalent sexually transmitted disease, over 80% of sexually active people are susceptible to its infection at some time in their life. It may possibly lead to variable illnesses in humans including genital warts, cervical cancer, and other malignancies. There are about 200 distinct forms of HPV, and these viruses are divided into two categories depending on their relationship with cancer: low-risk and high-risk (Heidegger et al., 2015).

Structure and Genome

HPV is a tiny, non-enveloped virus with an 8,000-base-pair circular ds-DNA makeup. The viral DNA is contained within an icosahedral capsid composed of two structural proteins, L1 and L2. The L1 protein is the principal structural component of the capsid and forms the virus's outer shell, whereas L2 is a minor structural protein found beneath the L1 shell. The capsid is made up of 72 capsomeres with T=7 icosahedral symmetry. HPV is roughly 50nm in diameter and lacks an envelope, making it resistant to physical and chemical agents. It consists of six functional proteins, E1, E2, E4, E5, E6, and E7, that perform various roles including DNA replication, gene translation, virion assembly and release, a membrane signaling protein, and cell cycle instability (Chakraborty et al., 2018).

Genotypes

HPV is classified into two subtypes based on its oncogenic potential: high-risk and low-risk. Low-risk HPV strains with a low probability of infection, such as types 6 and 11, have been

correlated with the emergence of genital warts as well as other benign lesions. High-risk strains of HPV, such as types 16 and 18, induce almost all of urological malignancies including cervical, anal, and penile, and oropharyngeal malignancy. HPV types 31, 33, 45, 52, and 58 are also categorized as high-risk (Salaria et al., 2022).

HPV Oncoproteins

HPV has various oncoproteins that are responsible for contributing to its oncogenic properties. The most recognized oncoproteins are E6 and E7. E6 engages with and suppresses the activity of tumor suppressor protein p53, which governs cell division and death. E7 interfaces with and inhibits the retinoblastoma protein (pRB), another significant tumor suppressor that modulates the cell cycle. Inhibition of these tumor suppressors can lead to uncontrolled cell proliferation ultimately causing cancer development (Bonab et al., 2021).

HPV-Induced Carcinomas

HPV is responsible for contributing 5.2% of all cancer cases (Salavatiha et al., 2021). It is widely acknowledged as the primary cause of nearly all cervical cancer cases (96%), with an estimated 570,000 newly diagnosed instances, having 311,000 mortality rate per year (Kessler, 2017)**.** Moreover, HPV is also accountable for the development of anal, penile, testicular, bladder, renal, and oropharyngeal cancers (Hodges et al., 2006; Wang et al., 2010). These malignancies are not prevalent, although their prevalence is growing in distinct areas of the world (Messa & Loregian, 2022).

Human Papillomavirus & Renal Cell Carcinoma

The association between HPV and various urological cancers including renal cell carcinoma (RCC) is still debated today due to conflicting results reported by different studies. The

literature is inconsistent on the involvement of HPV in the instigation of RCC due to variations in size of sample, tissue types, host genome, distinct regions, and techniques used to detect HPV (Sarier, 2021).

For example, in a PCR-based investigation of 28 renal cancer patients, HPV was not detected in any patient (Grce et al., 1997). Similarly, HPV was not identified in any of the 62 patients with renal tumors, leading the researchers to conclude that HPV was unlikely to have a carcinogenic effect in either benign or malignant renal tumors (Hodges et al., 2006). Contrary to expectations, HPV prevalence was found to be 52% when 56 individuals with renal cancer were analysed via in-situ hybridization (Kamel et al., 1994). Likewise, a small case-control investigation including 49 RCC cases resulted that the level of HPV prevalence was relatively 14.3% in the patients (Salehipoor et al., 2012). Other studies also demostrated contradictory conclusions relevant to the association of HPV in the RCC development (Farhadi et al., 2014; Khoury et al., 2013).

Renal Cell Carcinoma (RCC)

RCC is the most prominent kind of kidney tumor that originates from the tubular epithelium of the kidney. It is responsible for around 85% of all kidney cancers with an approximately 0.4 million new cases diagnosed each year. It is found to be more common in males as compared to the females, and it is most widespread in individuals age range from 50 to 70 years (Padala et al., 2020).

Epidemiology

The incidence rate of RCC varies among nations, with the greatest rates observed in developed countries. In the United States, RCC contributes roughly 3% of all adult malignancies and is the 7th prime cause of malignant mortality. Western and Northen Europe have the greatest

frequency of RCC. In Asia, the prevalence of RCC is lower as compared to Western countries, but it is elevating (Padala et al., 2020).

RCC is less prevalent in Pakistan than in Western countries, where it is responsible for approximately 3.2% of all cancers in the country. In Pakistan, RCC in males is the third common malignancy and in women, it is the fifth most frequent malignancy. Smoking, obesity, and heavy metal exposure are the top risk factors for RCC in Pakistan (Mahmood et al., 2021).

Histological Types

There are many histological types of RCC, including papillary, clear cell, chromophobe and collecting duct carcinoma. Clear cell RCC is the most common subtype, responsible for around 70-80% of the instances. Papillary RCC responsible for 10-15% of the instances, and chromophobe RCC accounts for 5% of instances. The prevalence of collecting duct carcinoma is less than 1%, hence it is considered as rare (Bahadoram et al., 2022).

Aim & Objectives

As there is no research particulars on the prevalence and specific genotype classification of HPV in RCC patients in the Pakistan the objectives of the present study are set as follows:

1. Collection of demographic data from healthy individuals and less- and more differentiated renal cell carcinoma patients in Pakistan.
2. Tissue sampling and collection of histopathological data from healthy individuals and less- and more differentiated renal cell carcinoma patients in Pakistan.
3. Establishing an etiological association between HPV and risk of developing renal cell carcinomas with respect to Pakistani population.

REVIEW OF LITERATURE

2.1 Human Papillomavirus (HPV)

HPV is a commonly sexually transmitted disease affecting both males and females. It only infects the epithelial cells. It is highly contagious and transmits through skin-to-skin contact, even in the presence of invisible warts or lesions. In some cases, HPV can also be transmitted through non-sexual means, such as from a mother to her newborn during childbirth, or through contaminated objects such as towels (Chakraborty et al., 2018). Majority of the HPV infections do not result any symptoms and go away on their own but they can also lead to serious health problems and cause genital warts or abnormal cell growth, which can further develop into different kinds of cancers such as cervical, anal, throat, and varuious other kinds of cancers. (Heidegger et al., 2015). Vaccination against HPV can provide significant protection against certain types of the virus and is recommended for both males and females to minimize the probability of HPV-related carcinomas and further different diseases (Chakraborty et al., 2018).

2.1.1. Genome

HPV is a small, non-enveloped, circular, and ds-DNA virus which is a member of the papillomaviridae family having a genetic makeup of approximately 8kb (Van Doorslaer et al., 2017). This genome consists of two types of regions; the coding region and the non-coding region. The coding region of the genome is made up of about 8 open reading frames (ORF) categorized into the early region and late region (Fig. 2.1). The ORF of the early region codes for six functional proteins such as E1, E2, E4, E5, E6, and E7 which are responsible for different functions for instances, replication of DNA, expression of genes, virion assembly and release, a membrane signaling protein, and cell cycle disruption (Chakraborty et al., 2018).

The viral proteins, E1 and E2, instigate replication of viral genome and perpetuate and segregate the genetic material of virus along with the cellular replication machinery. Furthermore, the proteins such as E5, E6, and E7 are requisited for escaping the response of host immune system and providing a promising cellular environment for the replication of viral DNA. Due to the modification of cellular escalation and differentiation, cells transform into carcinogens menacingly (Della et al., 2021). The proteins E6 and E7 of perilous HPV subtypes are usually responsible for causing genomic instability by interfering with the activity of tumor suppressor genes for instances, p53 and Rb, ultimately leading to cancer development (Bonab et al., 2021). The E5 protein permits epidermal cell differentiation and immune escape while E6 and E7 oncoproteins of HPV are associated with the effective deactivation of primary regulators of cell division, suppressor of cancer progression repressors, and activation of telomerase (Harężа et al., 2022).

Late region codes for L1 and L2 which are viral structural proteins that construct capsid (constituting 55-60nm size) around the genetic material of the virus. The capsid of most papillomaviruses is made of almost 72 capsomeres, known as monomer units of the capsid, arranged in regular icosahedral symmetry (Petrelli et al., 2016). L1 is the major protein involved in the composition of the capsid and its sequence varies significantly among all types of HPV while L2 is a minor protein and its sequence remains conserved (Schiller et al., 2010). Both of these structural proteins are involved in virion assembly and they interact with other macromolecules present in the cell that promote their entry into the host (Pappa et al., 2018). Non-coding region which is sited upstream of the early region and also termed as upstream regulatory region (URR). It comprised several cis-regulatory elements prerequisite for transcription and the origin of replication (Della et al., 2021).

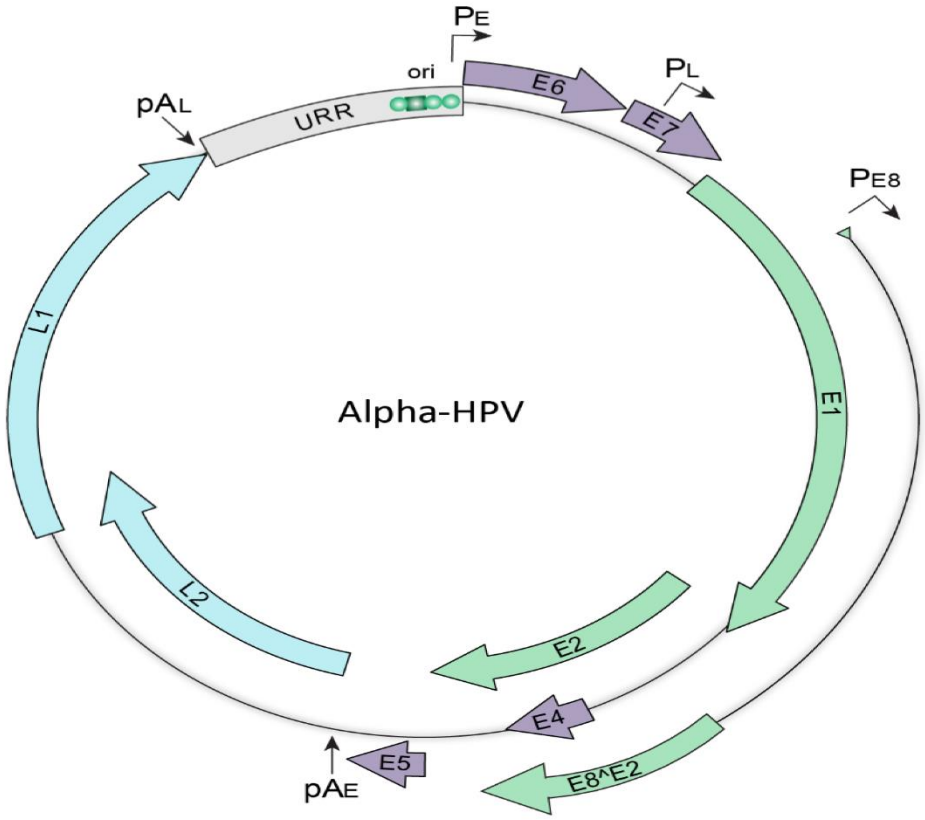


Figure 2.1: Structure of alpha genus human papillomavirus (α-HPV). The figure depicts the genetic composition of α-HPV, with green, blue, and purple arrows representing the early, late, and accessory viral ORFs, respectively. The URR appears in grey and comprises regulatory components such as the replication origin (ORI), containing binding sites for replication of E1 & E2 proteins (depicted as a green square & circles). The figure also depicts the early, late, and PE8 promoters, as well as the polyadenylation sites early (pAE) and late (pAL) (Della et al., 2021).

2.2 Life Cycle of Human Papillomavirus

For potent inflammation, HPV manipulates the transformation program of the layers of cutaneous and mucosal epithelia of the host. The virus ingresses the basal epidermal cells to trigger off the infection. After attachment to the basal lamina, a series of configurational changes are prompted to stimulate viral entry by endocytosis. L2 protein introduces the envelop surrounding the virus in a sac of membrane and leads to cytoplasmic trafficking. It also

expedites the transport of HPV-carrying vesicles to the host nucleus. After collapsing the nuclear surrounding in the course of mitosis, the virus infiltrates the nucleus. The HPV genome intertwined with host chromosomes until the termination of mitosis and remodeling of the nuclear membrane. The viral genome migrates towards promyelocytic leukemia nuclear bodies (PML-NBs) within the nucleus and endures limited cycles of DNA replication. During the initial stage of replication, the viral genome amplifies to create a limited number of extrachromosomal genetic material that will remain in the self-rejuvenating lower epithelial basal layer of cells. These affected basal layers of cells are the premise of skin warts and also function as a source of lasting chronic infection of HPV. The extrachromosomal genetic makeup of viruses leashes the host chromatin and preserves a sustained number of copies of the viral genome. As the epithelial differentiates, cells migrate to the upper epithelial membrane where the viral genome amplifies at a high rate. Late viral genes are expressed to congregate virions that cast off the epithelial lining in viral-loaded scales (Della et al., 2021).

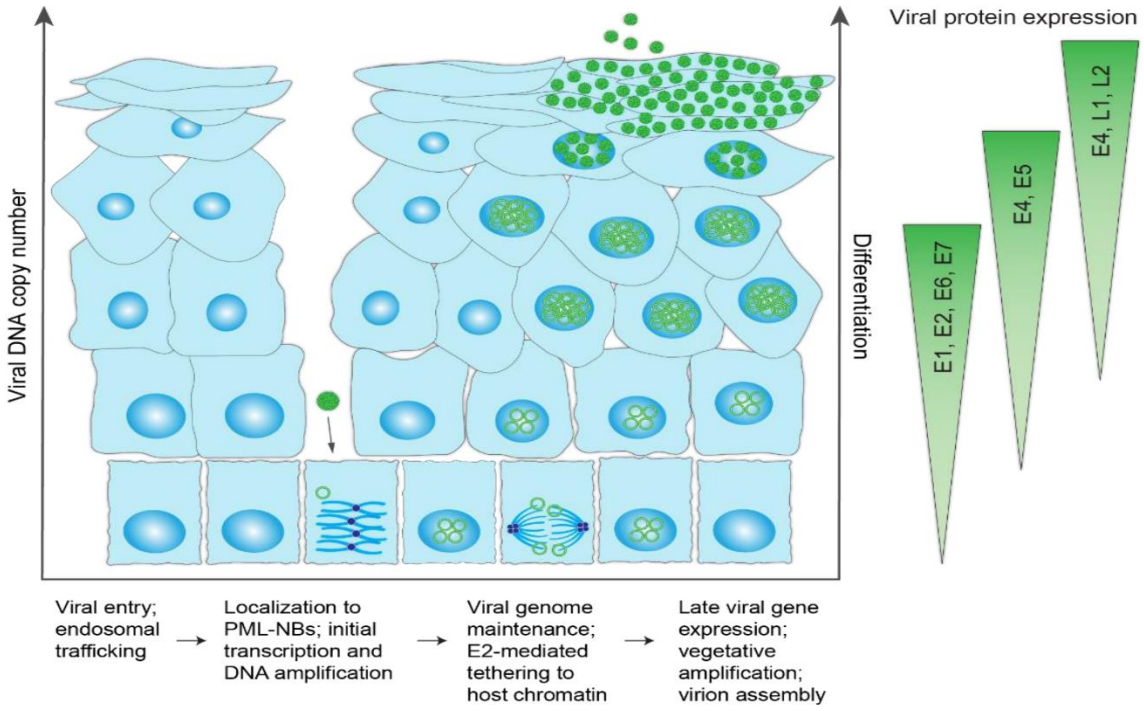


Figure 2.2: A diagrammatical representation of the human papillomavirus infectious life cycle (Della et al., 2021).

To maintain HPV-induced carcinomas, the E5, E6, and E7 oncoproteins change many different cellular signaling mechanisms including tumor protein p53, retinoblastoma protein (pRB), phosphoinositide 3-kinase (PI3K) / protein kinase B (PKB) / mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK) / epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK) / vascular endothelial growth factor (VEGF), epithelial-mesenchymal transition (EMT), Janus-kinase (JAK) / signal transducer and activator of transcription (STAT), and transforming growth factor beta (TGF-β) / tumor necrosis factor-alpha (TNF-α), for their necessity (Bonab et al., 2021). By interrelating HPV, micro-RNA (miRNA) also contributes in cell growth, migration, transformation, and apoptosis (Haręża et al., 2022).

2.3 Classification of Human Papillomavirus

More than 200 HPV serotypes have been discovered including high-risk and strains with a low level of risk depending on their cancerous potential (Salaria et al., 2022).

2.3.1. Low-risk Human Papillomavirus (LR-HPV)

LR-HPV strains usually cause asymptomatic infections which may disappear with the development of immunity in the human body, but can also cause warts on the penis, vulva, cervix, vagina, and sometimes in the mouth and throat. About ninety percent of these warts are caused by HPV-6 and -11 in comparison with other low-risk strains including 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 (Meites et al., 2021).

2.3.2. High-risk Human Papillomavirus (HR-HPV)

HR-HPV strains due to the high oncogenic potential are highly associated with invasive cancer development such as cervical, vulva, penis, vagina, anus as well as oropharyngeal cancers. HR-

HPV strains are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and others. Approximately 66% of cervical malignancy is induced by HPV-16 and HPV-18 globally (Meites et al., 2021).

2.3.3. Phylogenetic Grouping of Human Papillomavirus

The majority of HPV serotypes are classified under five main genera: Alphapapillomavirus, Betapapillomavirus, Gammapapillomavirus, Mupapillomavirus, and Nupapillomavirus. Each genus includes several HPV types that are categorized based on their genetic characteristics, clinical presentations, and risk of causing cancer or other diseases. Understanding the different types and genera of HPV is important for developing effective prevention and treatment strategies for HPV-related diseases (Della et al., 2021).

Alphapapillomavirus is the most studied genus and includes the high-risk types that are known to cause cancers such as cervical, anal, and head and neck cancer. HPV-containing Alpha genus induces infection on both cutaneous and mucosal layers of the epithelium. Betapapillomavirus includes the low-risk types that are associated with benign warts. Gammapapillomavirus and Mupapillomavirus include both high- and low-risk types and are less well-studied. Nupapillomavirus includes HPV types that infect non-human primates. HPV containing Beta, Gamma, Mu, or Nu genus induce infection on only cutaneous epithelium (Della et al., 2021).

2.3.4. Tropisms Grouping of Human Papillomavirus

They can also be grouped into two tropisms of cutaneous HPV types (cutHPV-1, -4, -8, -10, -38, -41, and -49) and mucosal HPV types (mucHPV-6, -11, -16, -18, -31, -33, -45, -52, and -58). Majority of the HPV subtypes infect the cutaneous epithelium while almost 40 different HPV subtypes infect the mucosal epithelium (Loenenbach et al., 2022).

2.4 Oncoproteins of Human Papillomavirus

The oncoproteins, E5, E6, and E7 can stimulate the metastasis of tumor cells. In carcinogenesis, stimulation, and suppression of cell transformation are related to the interactivity of HPV oncoproteins and cellular proteins of the host. These oncoproteins influence the escalation in the cell transformation process synergistically (Basukala & Banks, 2021).

2.4.1. E5 Oncoprotein

The protein, E5 is comprised of eighty-three amino acids with a molecular weight of 9.4kDa and 8.3kDa for HPV-16 and HPV-18, respectively. It is a quite small hydrophobic protein (Messa & Loregian, 2022) that resides at the transmembrane and is usually confined to intracellular membrane-enclosed organelles such as the Golgi apparatus and endoplasmic reticulum (L. Hu & Ceresa, 2009). The majority of the studies showed that E5 oncoprotein plays a key role in carcinogenesis, as it engages in mutating HPV-associated lesions. The latest studies have demonstrated its conspicuous significance in cell transmutation and regulation of the natural defense system and ultimately leads to tumor progression in cooperation with E7 and E6 (Estêvão et al., 2019).

By the inception of stimulating networks with growth factor receptors for instance with EFGR, E5 oncoprotein can instigate tumor cell amplification and result in sustained proliferation. Additionally, due to its position in the Golgi apparatus and the endoplasmic reticulum, E5 is important for regulation in cell signaling through its interaction with the ATPase which is a vacuolar proton that reduces endosomal acidulation. Endosomal acidification is a critical process that causes cell surface receptor regression; however, its dysregulation causes a reduction in cell surface receptors such as EGFR’s turnover, thus enhancing their signaling activity. Furthermore, the E5 oncoprotein can suppress the signaling cascade of fibroblast

growth factor receptor 2b / keratinocyte growth factor receptor (FGFR2b / KGFR) following abating autophagy (Ilahi & Bhatti, 2020). Corresponding to E6, the E5 oncoprotein incites a reduction in apoptosis and escalates the number of cells with aberrant genetic mutations, resulting in malignancy progression. By enhancing the degradation and removal of the pro-apoptotic protein Bax, E5 can also repress death (Estêvão et al., 2019; Haręża et al., 2022).

E5 contributes in tumor progression by overexpressing MET, a hepatocyte growth factor receptor (HGFR) (Scott et al., 2018). The indispensable role of oncoprotein the E5 is the regulation of the immune responses by interrelating with the major-histocompatibility complex class I (MHC-class I). Suppressed expressions of MHC class I can encourage immune escape by impeding cytotoxic T lymphocyte recognition of infected cells (Venuti et al., 2011). Substantially, despite possessing a leading role in cell transformation on its own, the E5 oncoprotein plays an additional role in tumor progression along with E6 and E7 (Basukala & Banks, 2021). Though, recent research demonstrates the oncogenic potential of E5, casting off the cover of oncoproteins including the E6 and E7 (Estêvão et al., 2019).

2.4.2. E6 Oncoprotein

Out of the three well-known oncoproteins, the E6 oncoprotein is highly related and responsible for cell transformation and metastasis of cells infected with HPV. E6 is composed of 160 amino acids possessing 18kDa of the total genome. This cysteine-rich protein is situated in the nucleus having two zinc-binding domains nearby 4 CXXC domains. It contributes to the establishment of different cancer-associated characteristics due to its involvement with several cell proteins of the host, disrupting normal important cellular activities (Barbosa et al., 1989).

The E6 oncoprotein's most significant function is to disrupt the functioning of the p53 tumor suppressor. The Ubiquitin-protein ligase E3A (UBE3A) which is familiar to an E6-Associated

Protein (E6-AP) acts similar to coupling link amidst E6 and p53, as E6 interacts with UBE3A conserved concurrence sequences (Haręża et al., 2022; Huibregtse & Beaudenon, 1996; Li et al., 2019). Although, E6 also disturbs different other motes that are involved in apoptosis in several ways. The E6 oncoprotein of the HR-HPV strain can mess with procaspase protein 8 (CASP8) and leads it to degradation (Messa & Loregian, 2022). It can also hinder TNF-induced cellular death by interacting with tumor necrosis factor receptor 1 (TNFR1) (Filippova et al., 2002). Nevertheless, E6 can repress the cellular death process by impeding the Fas/Fas ligand-mediated apoptosis pathway. E6 is also involved in the upregulation of nuclear factor kappa b (NF-κB) which suppresses apoptosis by stimulating the transcription of anti-apoptotic protein such as a apoptosis protein 2 (cIAP-2) suppressor (James, 2006; Reis et al., 2020).

Despite the high frequency of genetic mutations, the E6 oncoprotein has the potential to circumvent checkpoints and ultimately disrupt the cell cycle. E6 interferes and deactivates the transcriptional coactivator complex p300/CBP, elementary to modulating the cycle of the cell. It also performs a crucial part in cell cycle modulations mediated by miRNA regulations (Chiantore et al., 2016). For instance, the E6-AP-E6-p53 pathway-induced suppression of miR-34a results in the overexpression of the $p18^{Ink4c}$ and ingression the S-phase (Y. Zhu et al., 2018). E6 oncoprotein directly regulates the defense system through the transcription factor of interferon β (IFN-β), and interferon regulatory factor 3 (IRF-3), resulting in resist response of the immune system towards HPV antigens (Shah et al., 2015). The HR oncoprotein E6 prevents the activation of tyrosine kinase 2 (TYK2) through STAT/TYK2 pathway, disturbing the interferon α (IFNα) mediated immune responses (Gutiérrez-Hoya & Soto-Cruz, 2020). Additionally, E6 also disrupts the expressions of interferon κ (IFNκ), toll-like receptor-3 (TLR3), XIAP-associated factor 1 (XAF1), and p53, leading to abnormal cellular activities (Reiser et al., 2011).

The HR oncoprotein E6 individually upregulates the expression of the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3) and consequently generates genomic instability (Hatano et al., 2021). Moreover, it represses the repair protein of DNA, x-ray repair cross-complementing 1 (XRCC1), involve in base excision repair mechanisms and its repression enhances the genetic mutation rate. One of the indispensable roles of high-risk E6 oncoprotein is cell invasion by inducing the proteolytic breakdown of various tumor suppressor genes including the scribbled planar cell polarity protein, the par-3 family cell polarity regulator, the disc large MAGUK scaffold protein (Dgl), and the membrane-associated guanylate kinase 1, 2 and 3 (Estêvão et al., 2019; Facciuto et al., 2014; Kranjec et al., 2016). Moreover, it can also interfere with different adhesion proteins such as fibulin 1 and paxillin, and results in cell invasion and malignancy processes (Du et al., 2002; Wu et al., 2014).

2.4.3. E7 Oncoprotein

E7 oncoprotein was a pioneer to be discovered in all of the HPV oncoproteins. It is a small phosphoprotein composed of about one hundred amino acids. It consists of three conserved regions; CR1, CR2, and CR3. The CR1 region requires disrupting pRB and cellular oncogenic transformation. The CR2 region has a motif LXCXE which is involved in repressing tumor suppressor gene pRB activity. While the last region CR3 forms a zinc finger-like structure (Boulet et al., 2007). It had been discovered back in 1989 by Dyson and his co-workers the cancer causing protein E7, causes ubiquitination of pRB through cullin 2 including E3 ubiquitin ligases and ultimately degrades pRB which induces transcription factor E2F to be detached and downregulates cellular growth (Hwang et al., 2002). Consequently, this urges the activation of cyclin E and cyclin A of the S-phase and accumulation $p16^{INK4A}$, cyclin-dependent kinase inhibitor, (Z. Hu et al., 2014). Moreover, E7 can induce the degradation of E2F regulators such as p107 and p130 (DeCaprio, 2014).

The E7 oncoprotein can deactivate p21 & p27 cyclin inhibitors which are pivotal modulators for transition of the G1 phase into the S phase. Cellular growth is normally modulated by these proteins while their aberrant expressions retain the activity of cyclin-dependent kinase 2 (CDK2) and persuade abnormal and uncontrolled cell growth (Fischer et al., 2017). Though in the presence of impaired DNA, E7 promotes cellular growth by rising the turnover of claspin which is an essential checkpoint ATR-CHK1 for DNA damage signaling (Spardy et al., 2009). The E7 oncoprotein has also the potential to repress E2F6, the transcriptional repressor gene of the S-phase (McLaughlin-Drubin et al., 2008).

Correspondingly, the oncoproteins E6 & E7 can also interrupt interferon signaling as well as has the potential to suppress the toll-like receptor-9 (TLR9) (Hasan et al., 2013). Additionally, it can disrupt an essential element of the non-specific defense system, the cyclic GMP-AMP synthase- Stimulator of interferon genes (cGAS–STING) immune initiation cross talk that identifies the cytoplasmic DNA (Hatano et al., 2021). E7 oncoprotein contributes to tumor progression by activating and enhancing the expression of metalloproteinases causing extracellular matrix components degradation and ultimately tumor undergoes invasion (D. Zhu et al., 2015). Furthermore, the high-risk E7 oncoprotein downregulates the pRB activity and causes aberrant organization of centromeres resulting in accelerated genetic mutations. These genetic instabilities inevitably cause aneuploidy (Duensing et al., 2001).

2.5 Human Papillomavirus-Induced Carcinomas

HPV-caused infections usually recover within one or two years. The persistence of infection can also revert but over-time headways to cancer development (Meites et al., 2021). Each serotype of HPV causes a wide range of clinical and sub-clinical implications varying from symptomless infections to the formation of warts on the skin and genital area. Whereas, most

of the types of HPV are contemplated as a commensal part of the human microbiome (Van Doorslaer et al., 2017).

HPV infections are the second prime cause of cancer (Della et al., 2021). HR-HPV infections rise the probability of invasive cancer development. About ninety nine percent of cervical malignancy instances are induced by persistent genital HR-HPV infections (Okunade, 2020). Each year almost 0.69 million newly diagnosed cancer ascribed by HPV infections are reported globally. Out of which, 0.57 million (83%) are cervical cancer instances, among them 0.5 million (72%) are induced by HR-HPV serotypes 16 and 18, and 0.12 million (17%) are induced by LR-HPV serotypes 31, 33, 45, 52, and 58 (Della et al., 2021). HPV infections induce various cancers other than cervical cancer including anal (90%), vaginal (75%), oropharyngeal (72%), vulval (70%), and penile (60%) (Efua Sackey et al., 2022). The development of oropharynx, genital, and anus cancers in both women and men is exceptionally related to HPV infections (Van Doorslaer et al., 2017). Studies indicated the presence of HPV in most vaginal and vulvar tumors. 90% vaginal cancers are generally attributable to HPV infections. The genome and oncoproteins of HPV are also identified in ovarian tumor biopsies and fallopian tube specimens, although, its role in ovarian and endometrial invasiveness is still contentious (Della et al., 2021). The involvement of HPV has also been documented in the renal cell carcinoma development (Farhadi et al., 2014).

Table 2.1: Distribution of HPV serotypes in various carcinomas.

HPV Serotypes	Associated Cancers	Approximate Percentage
16, 18	Cervical	70%
16, 18, 31, 33, 45, 52, 58	Anal	85%
16, 18	Vulvar	40-50%
16, 18	Vaginal	70%

16, 18, 31, 33, 35, 52, 58	Oropharyngeal	90%
These percentages can vary depending on the population being studied and the testing methods used. Additionally, other HPV serotypes may also be involved in some cases (ICO, 2016).		

2.6 Renal Cell Carcinoma (RCC)

RCC is the frequent kind of adult renal cancer. It originates from the cells that line the kidney's tubules, which help filter blood and eliminate waste items. RCC can be undetectable in the initial stages and is frequently detected incidentally during imaging examinations for other purposes. Some variables enhance the likelihood of having this malignancy including smoking, obesity, hypertension, and certain hereditary diseases such as von Hippel-Lindau disease. Long-term dialysis, as well as exposure to chemical contaminants such as cadmium and organic solvents, have been associated to an elevated probability of developing RCC (Padala et al., 2020).

2.6.1. Epidemiology of Renal Cell Carcinoma

RCC responsible for around 85% of all kidney cancers. The frequency of RCC varies widely across the world, with the highest prevalence in the countries which are developed. In 2022, there were an estimated 403,000 emerging patients of kidney cancer and 179,360 deaths due to the RCC (Bukavina et al., 2022). In North America, Australia and Europe the incidence rates of RCC are approximately 10-15 per 100,000 individuals per year. In contrast, the incidence rates in Asia and Africa are much lower, at approximately 3-5 per 100,000 individuals per year. The prevalence of RCC has been on the rise over the past few years, which may be attributed to changes in lifestyle factors such as obesity & smoking (Padala et al., 2020).

In comparison to high-income nations, the incidence of RCC in Pakistan is quite low, with an approximate occurrence rate of 3.2% each year. Despite this, RCC is regarded as a major health issue in Pakistan, and its prevalence is anticipated to rise in the future years (Mahmood et al., 2021).

2.7 Histological Types of Renal Cell Carcinoma

RCC is categorized into several histological types, each with its unique set of characteristics and pathological implications. The most prevalent histological sub- classifications of RCC are clear cell, papillary, and chromophobe, contributing to approximately 75%, 10-15%, and 5% of all cases, respectively (Humera et al., 2015).

2.7.1. Clear Cell Renal Cell Carcinoma (ccRCC)

ccRCC is the prevalent RCC histological subtype, responsible for almost 75% of cases. On histological examination, the tumor cells have a translucent cytoplasm due to lipids and glycogen deposition within the cell. ccRCC is connected with variations in the Von Hippel-Lindau (VHL) gene, which governs cell proliferation and angiogenesis (Zhang & Ro, 2015).

2.7.2 Papillary Renal Cell Carcinoma (pRCC)

pRCC is the 2nd predominant histological sub-classification of RCC, contributing to around 10-15% of cases. The tumor cells show a papillary appearance on histology, with finger-like projections that penetrate into the renal tubules. Mutations in the MET oncogene, responsible for regulating cell proliferation and invasion, are associated to pRCC (Padala et al., 2020).

2.7.3. Chromophobe Renal Cell Carcinoma (chrRCC)

ChrRCC is infrequent histological form of RCC that is responsible for around 5% of the total number of instances. According to the histological examination, The cancer cells exhibit a distinct pale, eosinophilic cytoplasm with a distinct cell membrane. Changes in the genes that govern mitochondrial physiology and energy metabolism are correlated to chrRCC (Humera et al., 2015; Zhang & Ro, 2015).

2.7.4. Other Rare Subtypes

Collecting duct, medullary, and renal medullary carcinoma are all rare histological subtypes of RCC. Collecting duct carcinoma is an infrequent virulent subtype of RCC that develops from the epithelial cells of the kidney's collecting ducts. Medullary carcinoma is an infrequent kind of RCC linked to sickle cell trait or illness. Renal medullary carcinoma is a severe variant of RCC that most commonly affects younger people with sickle cell traits or illnesses (Humera et al., 2015; Zhang & Ro, 2015).

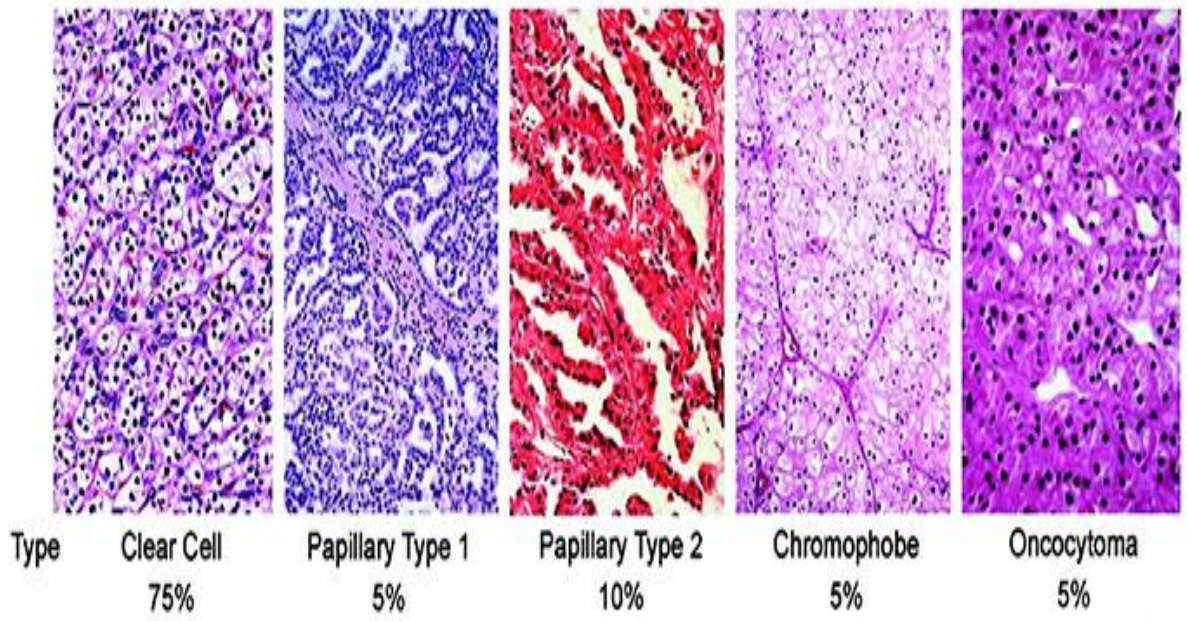


Figure 2.3: Prevalence of histological subtypes of renal cell carcinoma

(rarekidneycancer.org/blog/types-kidney-cancer, Jan 17, 2017).

2.8 Staging of Renal Cell Carcinoma

Staging and grading of cancer is an integral step for the assessment of the size and degree of spread of tumors. Standardization of cancer nomenclature facilitates investigating patterns and implications of therapeutic strategies on cancer management (Swami et al., 2019).

2.8.1. Tumor-Node-Metastasis (TNM) Staging

The widely acknowledged and most frequently employed staging method is the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging. It is recognized with its convenience of application, brevity, and reproducibility (Amin et al., 2017).

Table 2.2: TNM Staging of RCC according to AJCC 8th edition (Swami et al., 2019).

TNM Staging of Renal Cell Carcinoma		
Tumor (T)	Tx	Inaccessible to assess the primary tumor
	T0	Sign of primary tumor not observed
	T1	Tumor has a maximum diameter of about ≤7 cm, limiting to kidney
	T1a	Tumor has a maximum diameter of about ≤ 4cm, limiting to kidney
	T1b	Tumor has a maximum diameter of > 4 but ≤ 7cm, limiting to kidney
	T2	Tumor has a diameter of > 7cm, confined to kidney
	T2a	Tumor has a diameter of > 7cm but ≤ 10cm, limiting to kidney
	T2b	Tumor has a diameter of > 10cm, limited to the kidney
	T3	Tumor penetrates the most important veins or perinephral tissues but does not involve the ipsilateral adrenal gland, also cannot penetrate Gerota's fascia
	T3a	Tumor penetrates the perirenal and/or renal sinus fat, the pelvicalyceal system, or the renal vein or its segmental branches, but cannot penetrate Gerota's fascia
	T3b	Tumor substantially integrated the vena cava, beneath diaphragm
	T3c	Tumor aggressively invades vena cava wall or spreads into the vena cava atop the diaphragm
	T4	Tumor extends across Gerota’s fascia and may invades ipsilateral adrenal gland

Node (N)	Nx	Inaccessible to spread to localized lymph nodes
	N0	No sign of regional lymph node is observed
	N1	Localized lymph node is observed
	N2	It is not well defined
Metastasis (M)	M0	No sign of far away metastasis
	M1	Far away metastatis is observed

2.8.2. Stage Groupings

The of RCC is determined by analyzing the findings of a physical examination, a biopsy, and imaging investigations. As the T, N, and M categories are identified, their results are assembled through a procedure termed stage grouping to generate the overall stage of the malignancy (Bellmunt et al., 2014).

Table 2.3: Stage groupings of RCC according to AJCC 8th edition (Amin et al., 2017).

Stage I	T1a-1b N0 M0
Stage II	T2a-2b N0 M0
Stage III	T1a-3c N1 M0
	T3a-3c Nx-N0 M0
Stage IV	T4 any N M0
	any T any N M1

2.8.3. Grading

Tumor grading has a significant prognostic value, but its reliance on subjective microscopic interpretation and the extent of tumor collection poses certain challenges. The WHO system has positive impacts over the preceding Fuhrman grading system, and currently being used as standard reference for tumor grading (Warren & Harrison, 2018).

Table 2.4: Grading of RCC according to WHO (Moch, 2016).

Gx	Undetectable
G1	Basophilic and indistinct or missing nucleoli at a 40x objective
G2	Eosinophilic and noticeable nucleoli at 40x, but not seen at 10x objective
G3	Eosinophilic and prominent nucleoli at a 10x objective
G4	Multiple nuclei tumor large cells, significant nuclear pleomorphism, or rhabdoid / sarcomatoid differentiation

2.9 Diagnosis and Prognosis of Renal Cell Carcinoma

Renal Cell Carcinoma manifestations include hematuria, an abdominal lump or cyst, flank discomfort, weight loss, and exhaustion. Nevertheless, these symptoms may also be present in other illnesses, and RCC is often diagnosed with imaging techniques like CT or MRI scans. Once diagnosed, the therapeutic preferences for RCC are determined by the stage of the cancer, its size, and the diseased person's general well being.

The majority of treatment for localized RCC is surgery, either partial or radical nephrectomy. Ablative treatments for instance, radiofrequency or cryotherapy are also alternatives for tiny tumors in patients who are not surgical contestants. Targeted or immunotherapy is frequently used to treat advanced or metastatic RCC. The prognosis for RCC varies according to the stage of the tumor. The prognosis for early-stage RCC is positive, with the rate of survival of five years greater 90%. Advanced-stage RCC, on the other hand, has a substantially poorer

prognosis, with a 5-year rate of living fewer than 20%. Therefore, early detection and therapy are critical for improving RCC prediction (Padala et al., 2020).

MATERIALS AND METHODS

3.1 Ethical Declaration

This research was authorized as per Institutional Review Board (IRB no. 04-2021-02/14) of Atta-Ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan. All individuals provided informed consent before participating in this research. All the extraction procedures were carried out at Cancer Biology Laboratory, ASAB, NUST, Islamabad.

3.2 Chemicals, Plasticware, and Equipment

All the descriptive information of chemicals, enzymes, kit, primers, plasticware, and equipment used to conduct this research are given in the Tables 3.1, 3.2, 3.3, 3.4, and 3.5 respectively.

Table 3.1: Chemicals used in the study.

Chemicals	Company	Catalog No#
Absolute ethanol	Sigma-Aldrich	32221-M-2.5L
Agarose	Sigma-Aldrich	N/A
Chloroform	Sigma	UN1888
Distilled water	China	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	SLCG6707
Xylene	Merck	UN1307

Table 3.2: Enzymes and kit used in the study.

Products	**Company**	**Catalog No#**
DreamTaq^{TM} green PCR master mix (2x)	Thermo Fisher	K1081
DNA ladder 100 bp ready to load	Solis BioDyne	07-11-1950
DNA Loading Dye (6x)	Thermo Fisher	R0611
EvaGreen qPCR mix plus (5x)	Solis BioDyne	08-24-00001
QIAMP DNA FFPE ADVANCED KIT	Qiagen	56604

Table 3.3: Primers used in the study.

Primers	**Company**
Primers (β-globin)	Humanizing Genomics Macrogen
Primers (MY09/MY11)	Humanizing Genomics Macrogen

Table 3.4: Plasticware used in the study.

Plasticware	**Company / Manufacturer**
Aluminum foil	N/A
Centrifuge tubes (15ml)	Bio-fil
Centrifuge tubes (50ml)	Accumax
Disposable syringes	Uniject
EDTA blood vials	LABOVAC
Eppendorf reaction tubes (1.5ml)	USA
Eppendorf storage box	IMPORTED
Falcon tube racks (15ml)	China
Falcon tube racks (50ml)	China
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	China
PCR tubes	Orange Scientific
PCR tubes (0.2ml)	Fine Bio
PCR tube racks	USA
Plastic measuring cylinder (500ml)	China
Plastic measuring cylinder (1L)	China
Pipette tips (0.1-10µl)	Netherland
Pipette tips(10-100µl)	Netherland
Pipette tips (100-1000µl)	Netherland
Reagent Bottle (250ml)	Pyrex/SIMAX
Reagent Bottle (500ml)	Pyrex/SIMAX
Reagent Bottle (1000ml)	Pyrex/SIMAX

Table 3.5: Equipment used in the study.

Equipment	**Company**	**Model No./Serial No.**
Centrifuge machine	Sigma	136013
Deep freezer (-20^{o}C)	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	Colibri-Titertek Berthold	1122
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	N/A
Refrigerated centrifuge machine	Hermle (Germany)	66170035
Shaking incubator	K&K Scientific Supplier	K-SI 100R
UV transilluminator	Wealtec	ubw250551
Veriti thermal cycler	Applied Bio System	2990217863, 4375786
Vortex	Velp	F202A0173

3.3 Inclusion and Exclusion Criteria

Inclusion criteria for healthy individuals to conduct this quantitative cross-sectional study include both genders, male and female of age ranged 20-40 years, non-smoker / not drug user, without any co-morbidities, permanent resident of Pakistan who have granted informed consent. For RCC patients, males and females of any age group, who have undergone histopathological examination, and permanent residents of Pakistan who have granted informed consent and volunteered to provide a blood sample as well as tissue sample for HPV testing are included in the study.

Exclusion criteria for healthy individuals comprise below 20 and over 40 years of age group, smokers, drug users, having any of the co-morbidities, and not a resident of Pakistan. For RCC patients, who had previous records of other malignancy or chronic medical problems, and those who are not Pakistani residents.

3.4 Sample Size Calculation

The sample size is the number of participants either patients or other units of investigation that will be analyzed in a study in order to test the assumption being investigated. Calculating the sample size is mostly done to establish how many units are required to identify unknown clinical parameters, treatment effects, or associations following data collection. To conduct the

current prevalence study, Fisher's formula was used for the estimation of required RCC sample size (Daniel, 1999). The formula is given below;

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

Where,

n	Size of sample	Required to calculate
Z	Level of confidence	1.96 at 95% CI
P	Anticipated prevalence	3.2% = 0.032
d	Degree of accuracy / Precision	0.05

Calculation:

$$n = \frac{1.96^2\ (0.032)(1-0.032)}{0.05^2} = 47.5$$

It was concluded that with the prevalence of 3.2% (Mahmood et al., 2021) and 5% degree of accuracy under 95% confidence interval, the required sample size would be **48**.

3.5 Collection of Demographic Data

The demographic details were acquired with the informed consent of all subjects (both healthy and cancer patients). The data was collected using a prepared questionnaire centered on several factors such as age, gender, comorbidity, chronic kidney conditions, exposure to social drugs, etc to investigate and determine an association between these factors and RCC patients in

Pakistan. Whereas, the name and backgrounds of the patients and healthy subjects were kept confidential.

3.6 Sampling

As a control group, 50 control blood samples from healthy individuals and as an experimental group, 48 RCC tissue samples were included in this study.

3.6.1. Blood Samples Collection

Blood samples were collected in sterile ethylenediaminetetraacetic acid (EDTA) tubes from RCC patients and healthy individuals with informed consent. Control blood samples were obtained from healthy individuals. The samples were properly labeled with the identification number of the sample, date of collection, name, age, and gender of the patient. All the blood samples were reserved at -18°C until further processing.

3.6.2. Renal Cell Carcinoma Tissue Samples Collection

RCC formalin-fixed paraffin-embedded (FFPE) tissue specimens were obtained from the Department of Pathology, North West School of Medicine (NWSM), Hayatabad, Pakistan. They were stored at room temperature. Whereas, the fresh RCC biopsies were collected from the Department of Urology and Kidney Transplantation, Fauji Foundation Hospital (FFH), Rawalpindi, Pakistan. The biopsy samples were placed in a container containing 10% neutral buffered formalin. These biopsies were stored at 4°C. Moreover, the histopathological reports for both FFPE tissue and fresh biopsy samples were also collected specifying the histological types of RCC, TNM staging, and histological grading of tumor.

3.7 Genomic DNA Extraction

3.7.1. Extracting DNA from Blood Samples

DNA was extracted from whole blood samples. The procedure was performed by using the Phenol-Choloform method. This is a high-yielding protocol. Solutions A-D were previously prepared and optimized on other samples. The recipe for these solutions is given in the following table:

Table 3.6: Formulation of solutions for genomic DNA extraction from blood samples.

Sr. No #	Solutions	Composition	Quantity (for 500ml)
1.	**Solution A**	0.32M Sucrose	54.76g
		10mM Tris HCl (pH 7.5)	605.7mg
		5mM $MgCl_2$	238.028mg
		Autoclave above mixture. Add 1% (v/v) Triton X-100	5ml
2.	**Solution B**	10mM Tris HCl (pH 7.5)	605.7mg
		400mM NaCl	11.688g
		2mM EDTA (pH 8)	292.244mg
3.	**Solution C**	Phenol	-
4.	**Solution D**	Chloroform (24 vol)	480ml
		Isoamyl alcohol (1 vol)	20ml

In 2ml Eppendorf tubes, 500µl of blood and 500µl of Solution-A were added. The tubes were inverted 2 to 3 times and placed at ambient temperature for 5-10 minutes. The tubes were spun in the centrifuge at 13000rpm for 10 minutes followed by discarding of the supernatant. The obtained pellet was again solublized in 500µl of Solution-A and the and the eppendorfs were

spun at 13000rpm for 10 minutes after which the supernatant was discarded. Then nuclear pellet was dissolved in 400µl of Solution-B, 5µl of proteinase K, and 12µl of 20% SDS and incubated overnight at 37°C so that the proteinase K and 20% SDS inactivated the nucleases and denatured the proteins respectively.

After overnight incubation, 500µl of the freshly prepared combination of Solution-C and Solution-D was poured to each sample tube followed by thorough mixing. The tubes were spun in the centrifugation machine at 13000rpm for 10 minutes. After centrifugation, the aqueous layer was obtained in 1.5ml Eppendorf tubes and an equivalent volume to the collected aqueous layer Solution-D (300-500µl, varied per tube) was added. After this, centrifugation was carried out at 13000rpm for 10 minutes. Again aqueous layer was collected in 1.5ml Eppendorf tubes followed by the addition of 55µl of 3M sodium acetate (pH 6) along with chilled isopropanol (equivalent volume to the collection of the aqueous layer) for DNA precipitation. The tubes were repeatedly inverted to ensure the DNA precipitation and then centrifuged for another 10 minutes followed by the complete removal of the supernatant. Then 300µl of 70% ethanol was poured so as to obtain nuclear ball to dissolve and wash out all the salts. The tubes were centrifugated at 13000rpm for 7 minutes and then discarding the ethanol. Then the tubes were dried on the absorbent paper and the DNA pellet was dried by incubation at 37°C for 20-30 minutes. It was followed by the addition of 75µl of nuclease-free water to solubilize the DNA pellet.

3.7.2. DNA Extraction from Fresh Renal Cell Carcinoma Biopsy Samples

DNA extraction from fresh biopsy samples was also performed by using the Phenol-Choloform method.

In 2ml Eppendorf tubes, 50mg of fresh biopsy sample was taken. Before processing, the sample was washed with 100%, 70%, and 50% ethanol step by step to remove formalin

afterwhich centrifugation at 14000rpm for 5 minutes to decant ethanol was carried out. After washing, 500µl of Solution-A was added to each tube and the sample was subjected to homogenization by using Probe Sonicator (Hielscher) for 30 seconds. After homogenization, the phenol-chloroform protocol was followed to extract the DNA.

3.7.3. DNA Extraction from Formalin-Fixed Paraffin-Embedded Tissue Samples

Following the FFPE tissue collection, samples were sectioned, which included cutting 5-10 micrometers thin 3-5 sections and placing them in 2ml Eppendorf tubes.

DNA from FFPE tissue samples was extracted by using the Qiagen Qiamp DNA FFPE Advanced kit (Catalog number: 56604) as per the manufacturer's specified protocol. Whereas, manually DNA was extracted according to the protocol explained by (Pikor et al., 2011). The recipe for the digestion/lysis buffer is given in the following table:

Table 3.7: Composition of tissue digestion/lysis buffer.

Sr. No #	Tissue Digestion/Lysis Buffer	Quantity [for 100ml] (ml)
1.	1M Tris-HCl (pH 8.0)	4
2.	0.5M EDTA (pH 8.0)	0.2
3.	10% SDS	10
4.	Distilled water	80.8
5.	Proteinase K	5

The protocol consisted of three steps; deparaffinization, tissue digestion, and DNA extraction.

a) For deparaffinization, 1ml xylene was added to each tissue micro-sections containing tube and shaken at ambient temperature for 30 minutes to dissolve paraffin. Then the tubes were centrifugated at 14000rpm for 10 minutes at ambient temperature followed by decanting the supernatant. Repeated the steps of dissolving and decanting paraffin in xylene until the complete elimination of paraffin. Sections were washed with 100%, 70%, and 50% ethanol step by step to remove xylene followed by centrifugation at 14000rpm for 5 minutes to decant ethanol. Tissue pellets were air-dried.

b) After drying, 500µl of lysis buffer was added to each tube to facilitate the digestion of the tissue and denaturation of enzymes such as nucleases and proteins and overnight incubation was carried out at 50°C. Following incubation, all tubes were heated at 95°C for 10 minutes for making the proteinase K non-functional. 120µl of 6M NaCl was added in each tube and high-speed spinning was carried out at 14000rpm for 10 minutes so that the proteins were pelleted down. Then the supernatant was collected into another Eppendorf tube and 55µl of 3M sodium acetate (pH 6) was added and shaken vigorously by using a vortex mixer. For DNA precipitation, 600µl chilled isopropyl alcohol (IPA) was added to each tube and then incubated overnight at -20°C.

c) As the incubation period was over, DNA suspensions in IPA were high speed spun at 14000rpm for 20 minutes at -4°C followed by discarding the supernatant. Then 300µl of 70% ethanol was added to dissolve and wash out all the salts. The tubes were centrifugated at 13000rpm for 7 minutes followed by discarding the ethanol. Then the tubes were dried on the absorbent paper and the DNA pellet was dried by incubation at 37°C for 20-30 minutes. It was followed by the addition of 75µl of nuclease-free water to solubilize the DNA pellet.

3.8 DNA Quantification

DNA yield was determined using the nanodrop spectrophotometer before storing the samples at -20°C. A spectrophotometer can ascertain the purity and concentration of a very minute quantity of DNA sample.

On the pedestal, 1µl of each DNA sample was loaded and the absorbance was measured. The nanodrop software computes the DNA concentration based on the obtained absorbance values.

The absorbance of DNA is maximum near 260nm. The A260/280 ratio is a purity indicator of DNA. 1.8 or higher A260/280 ratio indicates pure DNA samples. The samples that had a sufficient quantity of DNA were further used for PCR amplification.

3.9 1% Agarose Gel Electrophoresis

For the preparation of 1% gel, 100ml of 1x Tris-acetate-EDTA (TAE) buffer was taken in a long cylindrical flask. Then 1g of agarose was added for solidification. The suspension was heated in the microwave oven and then incubated at room temperature for 3-5 minutes. 5µl of ethidium bromide which is an intercalating dye that binds to ds-DNA and glows when seen under a Ultra-violet (UV) Transilluminator was added to the gel mixture for pre-staining.

The mixture was poured into the gel tray followed by adjusting the gelling comb into the tray and then cooled at room temperature until it solidified in the form of a gel. As the solution solidified, the gelling comb was removed and the 1µl loading dye mixed with the 9µl DNA sample was loaded into the wells in the gel. Then gel electrophoresis machine was turned ON with black (-ve) and red (+ve) electrodes at 80V for 20-25 minutes.

The DNA bands were observed by using a UV Transilluminator.

3.10 Amplification of Housekeeping Gene

Extracted genomic DNA was confirmed by amplifying the human housekeeping gene, beta-globin (β-globin) with 110bp amplicon size. Primer used for the amplification of housekeeping gene are mentioned in the given Table 3.10. For amplification of housekeeping genes, the PCR reaction mixture of 20µl volume was prepared in a sterile microcentrifuge tube. 10µl of DreamTaq^{TM} Green PCR Master Mix (2X) (Thermofisher) added to the tube after which there was addition of 6µl nuclease-free water, 1µl of forward primer, and 1µl of reverse primer (Eurofins | Genomics). Then 2µl of DNA template added to the PCR reaction mixture (Table 3.8). A reaction mixture without a DNA template was prepared to be taken as a negative control (2µl more of NF water was added instead of the DNA template).

Table 3.8: PCR single reaction mixture recipe for amplification of housekeeping gene.

Components	Concentrations (µl)
DreamTaq^{TM} Green PCR Master Mix	10
PCR – Water	6
DNA Template	2
Primer (Forward)	1
Primer (Reverse)	1
Total Concentration	20

The contents of the reaction were mixed by short-spinnings on a micro-centrifuge machine for 30 seconds. The reaction tubes were placed in a Veriti Thermal Cycler (Applied Biosciences), and the PCR was carried out on the following conditions: Initial denaturation: 95°C for 5 minutes; denaturation: 95°C for 30 seconds; annealing: 57°C for 35 seconds; extension: 72°C for 30 seconds; repeated steps 2-4 for 35 cycles; final extension: 72°C for 7 minutes; Hold 4°C

(Figure 3.1). As the PCR completed, the PCR vials taken out from the thermal cycler, and 12µl of the amplified product was used for confirmation by 2% agarose gel electrophoresis.

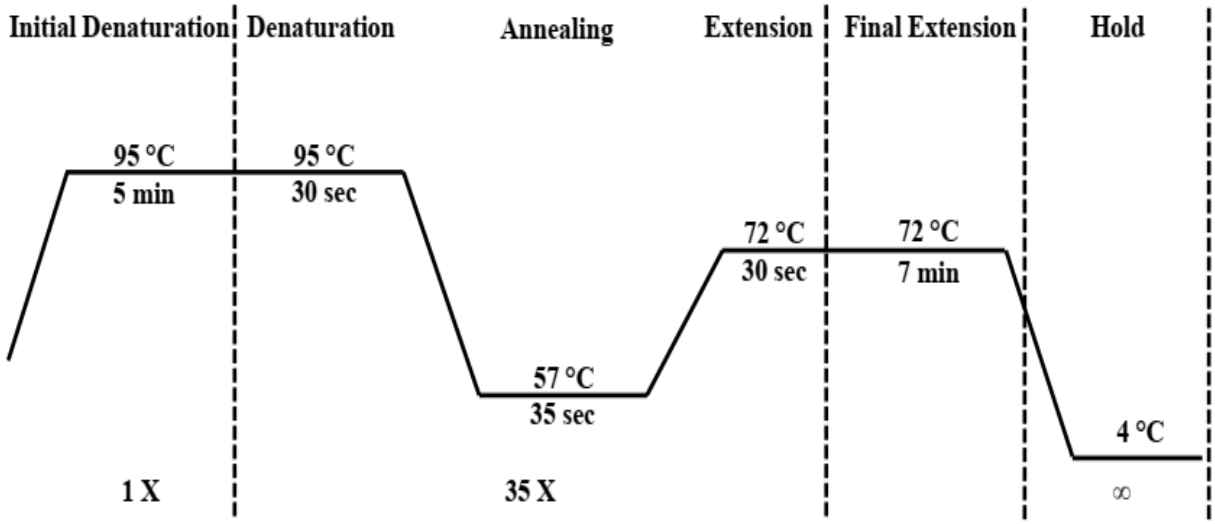


Figure 3.1: PCR amplification profile for housekeeping gene.

3.11 2% Agarose Gel Electrophoresis

For the preparation of 2% gel, 100ml of 1x TAE buffer was taken in a long cylindrical flask. Then 2g of agarose was added for solidification. The suspension was heated in the microwave oven and then incubated at room temperature for 3-5 minutes. 5µl of ethidium bromide which is an intercalating dye that binds to ds-DNA and glows when seen under a UV Transilluminator was added to the gel mixture for pre-staining. The mixture was poured into the gel tray followed by adjusting the gelling comb into the tray and then cooled at room temperature until it solidified in the form of a gel.

As the solution solidified, the gelling comb was removed and the 12µl PCR product was loaded into the wells in the gel. After that 3µl of 100bp DNA ladder (Solis Biodyne) was loaded in a separate well at the start of each lane. Then gel electrophoresis machine was turned ON with black (-ve) and red (+ve) electrodes at 80V for 45 minutes. The DNA bands were observed by using a UV Transilluminator.

3.12 Detection of HPV via Conventional PCR

Following the amplification of housekeeping gene, The annealing temperature of HPV consensus primer MY09/MY11 (452bp) amplifying the conserved L1 region was optimized by performing gradient PCR using plasmid. Plasmid was designed to be used as HPV positive control. For this purpose, a short region of L1 was cloned in pMVvectors. The details of HPV DNA sections and plasmid sequences are given in Table 3.9.

After optimization, the presence of HPV in RCC tissue samples was evaluated. Primer details used for the detection of HPV via conventional PCR are mentioned in the given Table 3.10.

Table 3.9: Details of plasmids.

Plasmid	Sequence 5′-3′	Length (bp)	Plasmid Type
HPV (L1)	ataaggcacagggtcataacaatggtgtttgctggcataatcaattatttgttact gtggtagataccactcgcagtaccaatttaacaatatgtgcttctacacagtctc ctgtacctgggcaatatgatgctaccaaatttaagcagtatagcagacatgttga ggaatatgatttgcagtttatttttcagttgtgtactattactttaactgcagatgttat gtcctatattcatagtatgaatagcagtattttagaggattggaactttggtgttcc ccccccgccaactactagtttggtggatacatatcgttttgtacaatctgttgctat tacctgtcaaaaggatgctgcaccggctgaaaataaggatccctatgataagtt aaagttttggaatgtggatttaaaggaaaagttttctttagacttagatcaatatcc ccttggacgtaaatttt	468	pMV

Table 3.10: Primers used for amplification of housekeeping gene and detection of HPV in RCC tissue samples.

Primers		Sequence 5′-3′	Target gene	Amplicon size (bp)	Annealing Temp. (°C)
β-globin	F	ACACAACTGTGTTCACTAGC	β-globin	110	57
	R	CAACTTCATCCACGTTCACC			
MY09/MY11	F	CGTCCMARRGGAWACTGATC	L1	452	55
	R	GCMCAGGGWCATAAYAATGG			

For the detection of HPV, the PCR reaction mixture of 25µl volume was prepared in a sterile microcentrifuge tube. 12.5µl of DreamTaq^{TM} Green PCR Master Mix (2X) (Thermofisher) added to the tube followed by the addition of 8.5µl nuclease-free water, 1µl of forward primer, and 1µl of reverse primer. Then 2µl of DNA template added to the PCR reaction mixture (Table 3.11). A reaction mixture was prepared to be taken as a positive control (1µl of plasmid and 1µl more of NF water was added).

Table 3.11: PCR single reaction mixture recipe for detection of HPV.

Components	Concentrations (µl)
DreamTaq^{TM} Green PCR Master Mix	12.5
PCR – Water	8.5
DNA Template	2
Primer (Forward)	1
Primer (Reverse)	1
Total Concentration	25

The contents of the reaction were mixed by short-spinnings on a micro-centrifuge machine for 30 seconds. The reaction tubes were placed in a Veriti Thermal Cycler (Applied Biosciences), and the PCR was carried out on the following conditions: Initial denaturation: 95°C for 5 minutes; denaturation: 94°C for 30 seconds; annealing: 55°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 (Figure 3.2). As the PCR completed, the PCR vials were taken out of the thermal cycler, and 12µl of the amplified product was taken for confirmation by 2% agarose gel electrophoresis.

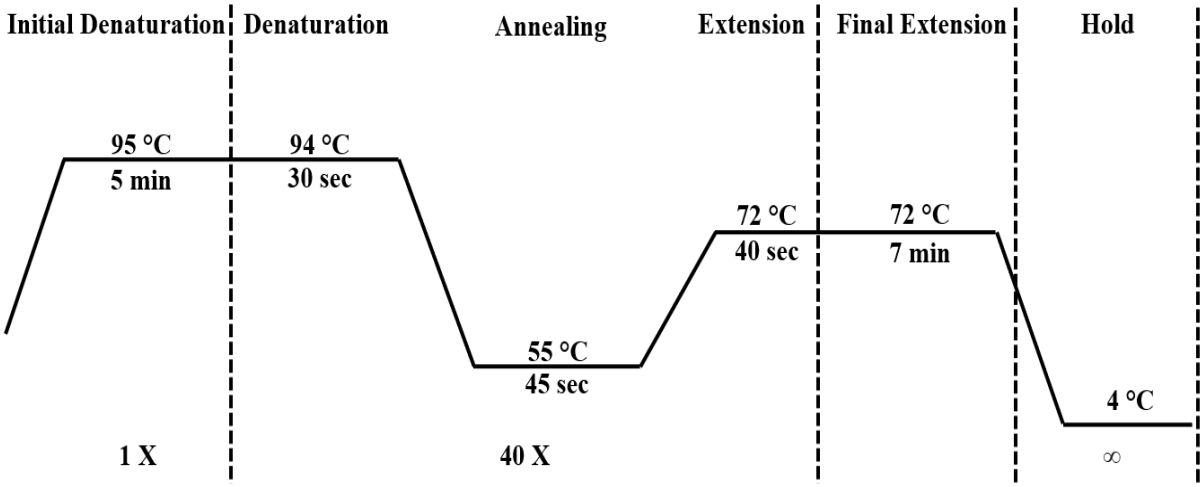


Figure 3.2: Optimized PCR amplification profile for HPV consensus primer MY09/MY11.

3.13 2% Agarose Gel Electrophoresis

For the preparation of 2% gel, 100ml of 1x TAE buffer was taken in a long cylindrical flask. Then 2g of agarose was added for solidification. The suspension was heated in the microwave oven and then incubated at room temperature for 3-5 minutes. 5µl of ethidium bromide which is an intercalating dye that binds to ds-DNA and glows when seen under a UV Transilluminator was added to the gel mixture for pre-staining. The mixture was poured into the gel tray followed by adjusting the gelling comb into the tray and then cooled at room temperature until it solidified in the form of a gel. As the solution solidified, the gelling comb was removed and the 12µl of PCR product was loaded into the wells in the gel. After that 3µl of 100bp DNA ladder (Solis Biodyne) was loaded in a separate well at the start of each lane. Then gel electrophoresis machine was turned ON with black (-ve) and red (+ve) electrodes at 80V for 45 minutes. The DNA bands were observed by using a UV Transilluminator.

3.14 Statistical Analysis

Analyis was performed on the obtained data by using the GraphPad Prism version 8. The clinical and histopathological features were assessed by using Chi square test, and statistical

multivariate analysis, where appropriate. Differences were considered to be statistically significant when P-values were less than 0.05.

RESULTS

4.1 Baseline Characteristics

4.1.1. Characteristics of Healthy Individuals

As a control, 50 blood samples were collected from healthy individuals included 28 males and 22 females (Table 4.1). The healthy individual's age ranged from 20 to 40 years, age of 74% individuals ranged 20-30 while 26% were in age range of 31-40. Seventy two percent individuals weighed 45-70kg and 28% weighed 71-95 kg. Marital status of 22% was married while 78% unmarried but majority of healthy individuals (82%) were educated, 18% were uneducated. Twenty percent of individuals had active lifestyle, 76% medium active and only 4% had less active. Majority (94%) were living in urban areas while only six percent living in rural areas.

All healthy individuals were non-smokers, 90 % were non-allergic while only two percent were dust allergic. All individuals have no co-morbidities.

Table 4.1: Characteristics of healthy individuals in Pakistan.

Variables/Parameters Measured		**Healthy Individuals (n=50)**	**Percentage (%)**
Personal Information			
Gender	Male	28	56
	Female	22	44
Age (years)	20-30	37	74
	31-40	13	26
Weight (kg)	45-70	36	72
	71-95	14	28
Marital Status	Married	11	22
	Unmarried	39	78
Education	Educated	41	82
	Uneducated	09	18

Variables/Parameters Measured		Healthy Individuals (n=50)	Percentage (%)
Lifestyle	Active	10	20
	Medium active	38	76
	Less active	02	04
Residential Area	Rural	03	06
	Urban	47	94
Clinical Information			
Smoking / Alcohol Addicted	Male	00	00
	Female	00	00
Allergic Status	Dust	02	04
	Pollen	00	00
	Dust & Pollen	03	06
	Non-allergic	45	90
Co-morbidities	Diabetes	00	00
	Hypertension	00	00
	Other	00	00

n stands for number of healthy individuals participated in the study.

4.1.2. Characteristics of Renal Cell Carcinoma Patients in Pakistan

This study included 48 tumor tissue samples from RCC patients. Clinical and pathological information was also collected. Out of 48 RCC patients, 30 were males and 18 females (Table 4.2). The patients' age ranged from below 50 to over 80 years, age of 44% patients was below 51 year, 40% patients were between 51 and 70 years while 16% were between 71-90 years of age. Figure 4.1 depicts the percentage of gender distribution of RCC patients in Pakistani population falling under different age groups. According to this chart, approximately 63% individuals were male RCC patients including 43.3% of them were falling under ≤ 50 age group whereas, 37% individuals were female RCC patients including 50% of them were falling under 51-70 age group. The incidence rate of RCC appeared higher in males as compared to females with a ratio of 2:1.

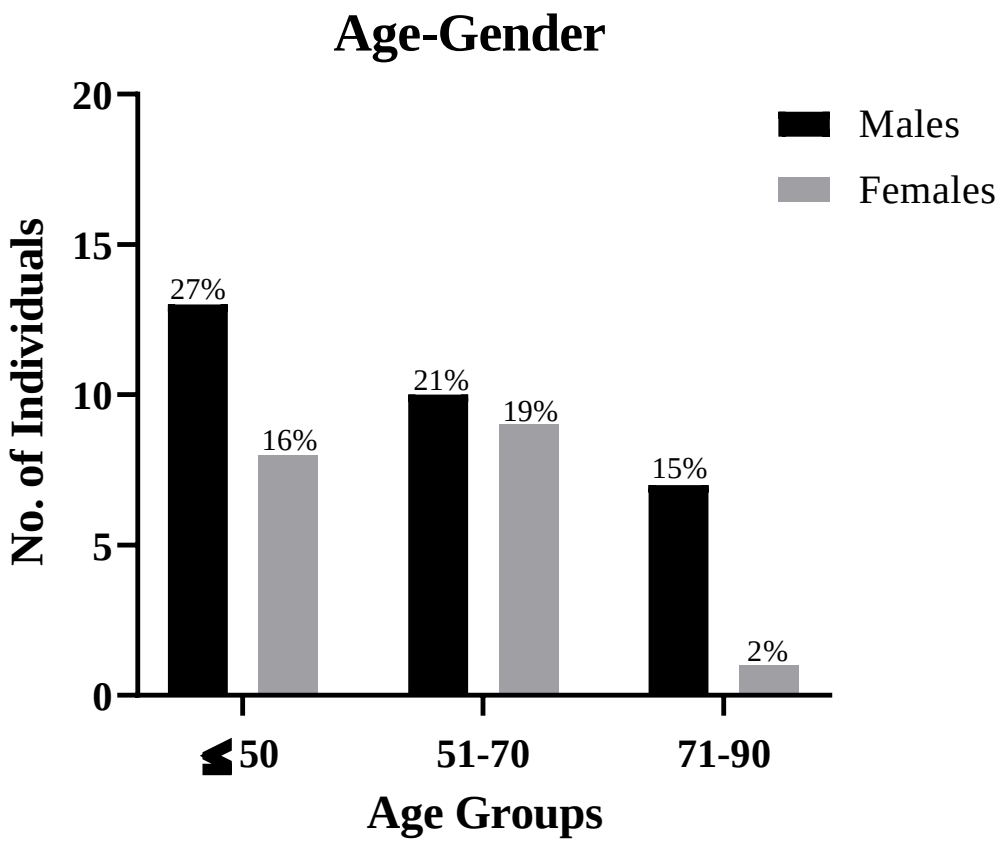


Figure 4.1: Gender distribution categorizing into different age groups of renal cell carcinoma patients in Pakistan. Out of total 48 RCC patients 63% were males and 37% females.

Twenty seven percent of RCC patients were married, four percent unmarried. Four percent of RCC patients were educated, 27% uneducated. Out of 48 RCC patients, 21% had active lifestyles, 2% medium active and 8% less active lifestyle. Ten percent of RCC patients were living in rural areas while 21% in the urban areas. The data about marital status, education, lifestyle, residence were not available for majority of RCC patients (69%).

Four percent of 48 RCC patients were occassional smokers, 27% never smoke while no one was reported regular smoker, however, data of 69% RCC patients was not available. The co-morbidities features of RCC patients showed that no patient has been observed as sole diabetic, 2% patients had hypertension, 6% had both diabetes and hypertension, 8% had other co-morbidities and 6% had no co-morbidities.

Table 4.2: Basic and clinical characteristics of RCC patients in Pakistan.

Characteristics		**No. of RCC Patients (N=48)**	**Percentage (%)**
Gender	Male	30	63
	Female	18	37
Age (years)	≤ 50	21	44
	51-70	19	40
	71-90	08	16
Marital Status	Married	13	27
	Unmarried	02	04
	NA	33	69
Education	Educated	02	04
	Uneducated	13	27
	NA	33	69
Lifestyle	Active	10	21
	Medium active	01	02
	Less active	04	08
	NA	33	69
Residential Area	Rural	05	10
	Urban	10	21
	NA	33	69
Clinical Information			
Smoking	Never	13	27
	Sometimes	02	04
	Regularly	00	00
	NA	33	69
Co-morbidities	Diabetes	00	00
	Hypertension	01	02
	Both	03	06
	Other	04	08
	None	03	06
	NA	37	77

N stands for number of renal cell carcinoma's patients participated in the study; **NA** stands for not available.

The comparison of healthy and RCC patient's characteristics showed significant differences for measured basic and clinical parameters except gender which was non-significant (Table 4.3).

Table 4.3: Comparison of characteristics of healthy individuals and renal cell carcinoma (RCC) patients in Pakistan.

Variables/Parameters Measured		Healthy Individuals (n=50)	RCC Patients (N=48)	SE	P Value
Personal Information					
Gender	Male	28	30	1,0	0.5128
	Female	22	18	2,0	
Marital Status	Married	11	13	1,0	<0.0001
	Unmarried	39	02	18,5	
	NA	00	33	16,5	
Education	Educated	41	02	19,5	<0.0001
	Uneducated	09	13	2,0	
	NA	00	33	16,5	
Lifestyle	Active	10	10	0,0	<0.0001
	Medium active	38	01	18,5	
	Less active	02	04	1,0	
	NA	00	33	16,5	
Residential Area	Rural	03	05	1,0	<0.0001
	Urban	47	10	18,5	
	NA	00	33	16,5	
Clinical Information					
Smoking	Smokers	00	02	1,0	<0.0001
	Non-smokers	00	13	6,5	
	NA	00	33	16,5	
Co-morbidities	Hypertension	00	01	0,5	0.0011
	Diabetes + Hypertension	00	03	1,5	
	Other	00	04	2,0	
	None	50	03	23,5	
	NA	00	37	18,5	

n stands for number of healthy individuals; **N** stands for number of renal cell carcinoma's patients participated in the study; **NA** stands for not available; **SE** stands for standard error; **P** value <0.05 considered as significant.

Only two percent of RCC patients had family history with malignancy, 29% didn't have family history of malignancy while data were not available for 69% of patients (Table 4.4). Right-sided RCC was in 54% patients while left-sided in 46% patients.

Table 4.4: Family history, tumor laterality and histological characteristics of RCC patients in Pakistan.

Characteristics		No. of RCC Patients (N=48)	Percentage (%)
Family History with Malignancies	Yes	01	02
	No	14	29
	NA	33	69
Tumor Laterality	Left	22	46
	Right	26	54
Histological Types	Clear Cell	39	81
	Papillary	03	07
	Chromophobic	03	06
	Granular	01	02
	NA	02	04

N stands for number of renal cell carcinoma's patients participated in the study.
NA stands for not available.

Histological types of RCC including clear cell renal cell carcinoma was dominantly observed in 81% cases while papillary and chromophobe renal cell carcinoma were observed in 7% and 6% of cases respectively whereas granular renal cell carcinoma was observed in 2% of cases (Figure 4.2).

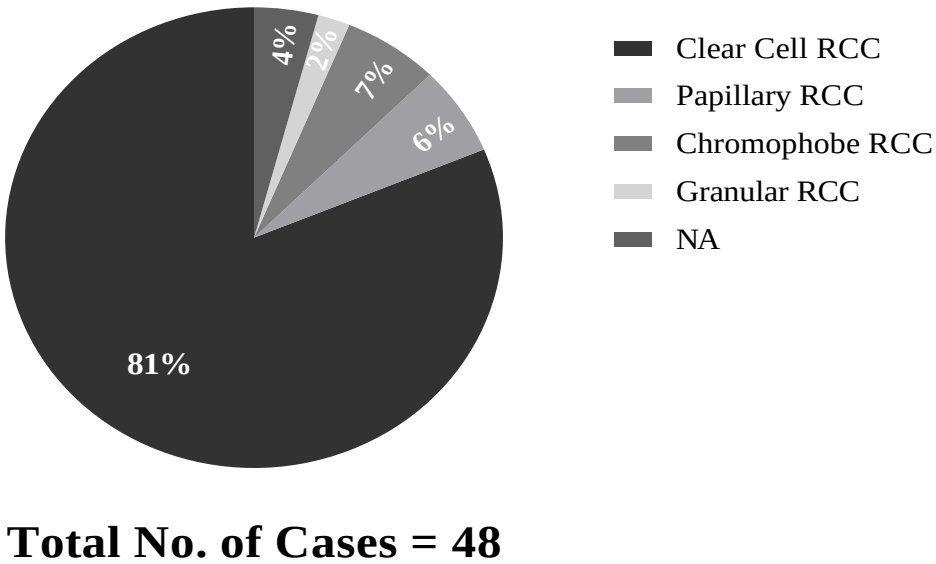


Figure 4.2: Histological subtypes distribution of RCC.

According to RCC histological grading (WHO), 38% of cases were detected at Grade 2 and 31% of cases were diagnosed at Grade 1 whereas 25% of the cases were detected at Grade 3 followed by Grade 4 (4%). It was also observed that most of the cases were diagnosed at Stage II (38%) and 44.4% of these cases were dominantly categorized under Grade 2, comprised of 16.7% of total Stage II cases. Among total, 33% of cases were diagnosed at Stage III dominantly categorizing under Grade 3 comprising 43.8% of total Stage III cases. 27% of cases were diagnosed at Stage I categorizing under Grade 1 (14.6%) comprising of 54% of Stage I cases and Grade 2 (10.4%) comprising of 38.5% of Stage I cases (Figure 4.3).

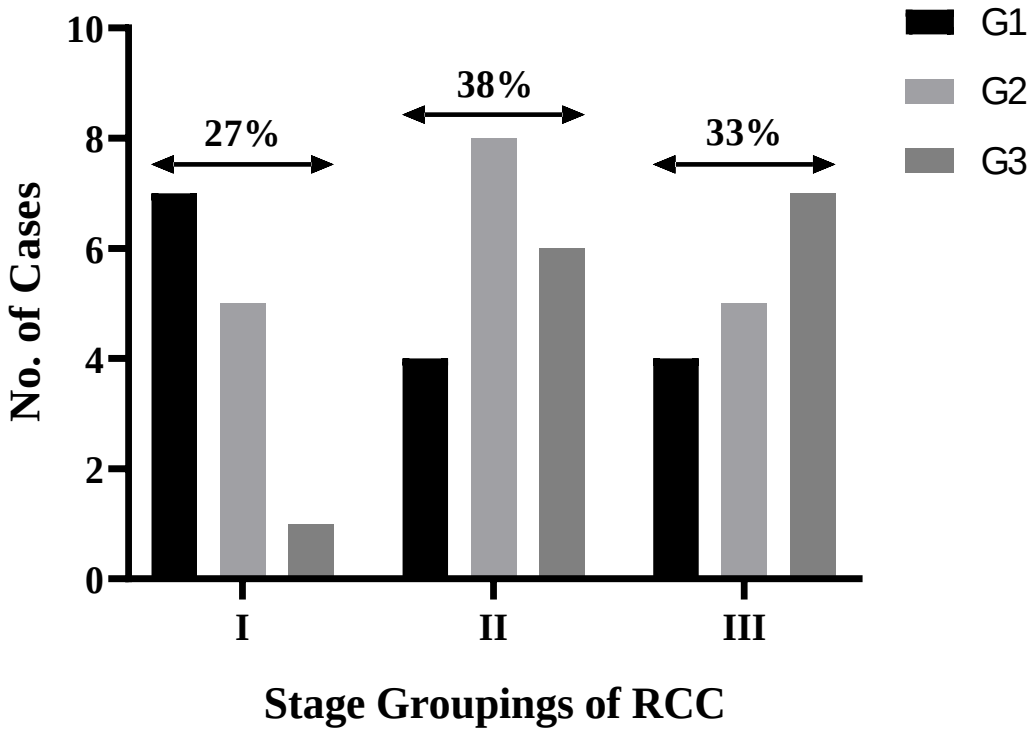


Figure 4.3: Distribution of RCC histological grading categorized into stage groups of renal cell carcinoma.

The TNM stages of RCC patients are given in Table 4.5. The tumor T2 was most frequent (35%) followed by T3 (33%) and T1 (29%) while T0 and T4 were not noted. All samples had Nx Node and M0 Metastasis.

Table 4.5: TNM staging of RCC.

Characteristics		**No. of RCC Patients (N=48)**	**Percentage (%)**
Tumor (T)	Tx	01	02
	T0	00	00
	T1	14	29
	T2	17	35
	T3	16	33
	T4	00	00
Node (N)	Nx	48	100
	N0	00	00
	N1	00	00
	N2	00	00
Metastasis (M)	M0	48	100
	M1	00	00
Stage Grouping	I	13	27
	II	18	38
	III	16	33
	IV	00	00
	NA	01	02
Histological Grade (WHO/ISUP)	G1	15	31
	G2	18	38
	G3	12	25
	G4	02	04
	Gx	01	02

N stands for number of renal cell carcinoma's patients participated in the study; **NA** stands for not available.

4.2 Validation of Extracted Genomic DNA

DNA extraction was done from blood samples of healthy individuals and RCC tissue samples, using the Phenol-Chloroform extraction method (details are provided in Chapter 3). Figure 4.4 & 4.5 depicts validation of extracted genomic DNA from control blood and RCC tissue samples respectively by running 1% agarose gel electrophoresis at 80V for 15min.

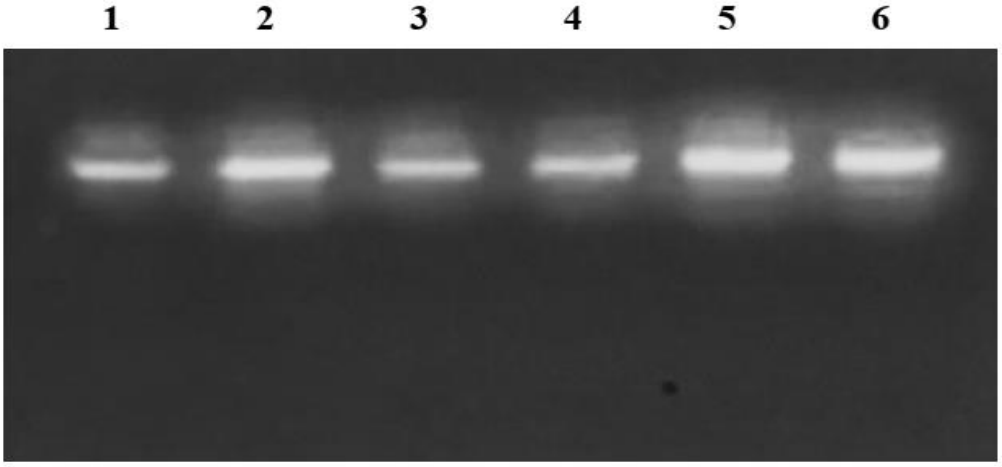
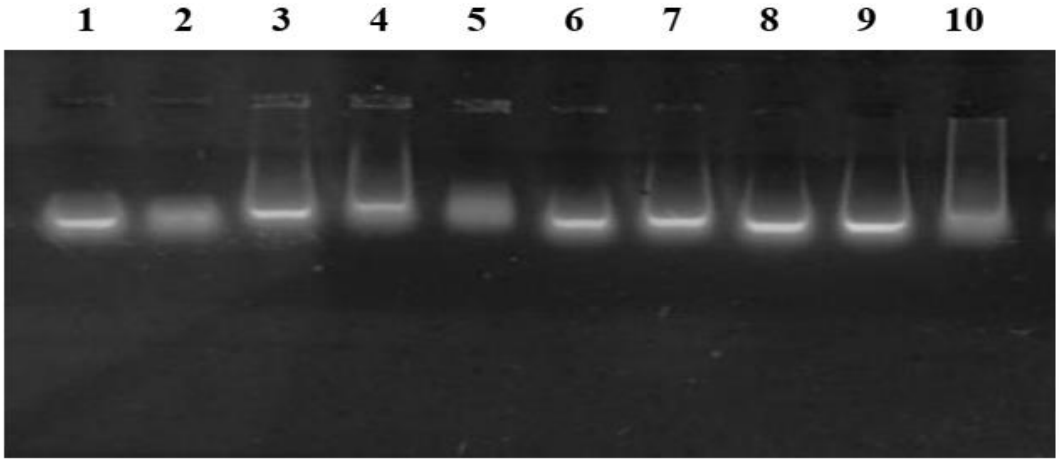


Figure 4.4: Demonstration of validation of extracted genomic DNA from control blood samples via 1% agarose gel electrophoresis (80V, 15min).

a.



b.

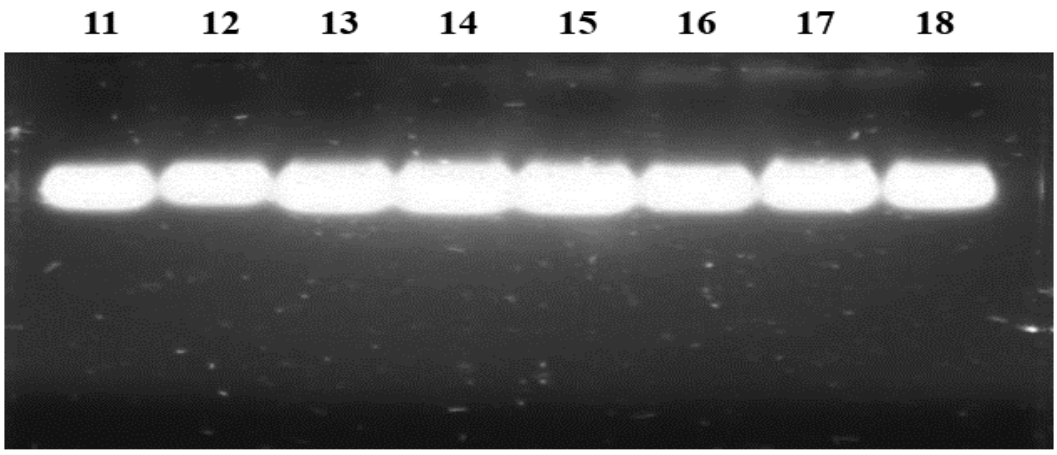


Figure 4.5 (a & b): Demonstration of validation of extracted genomic DNA from RCC tissue samples via 1% agarose gel electrophoresis (80V, 15min).

4.3 Validation of Amplified Housekeeping Gene

The integrity of isolated DNA was confirmed by amplifying human housekeeping genes, β-globin and β-actin. All DNA samples successfully amplified 110bp of β-globin. Figure 4.6 represents the validation of amplified of housekeeping gene by running 2% agarose gel electrophoresis at 80V for 45min.

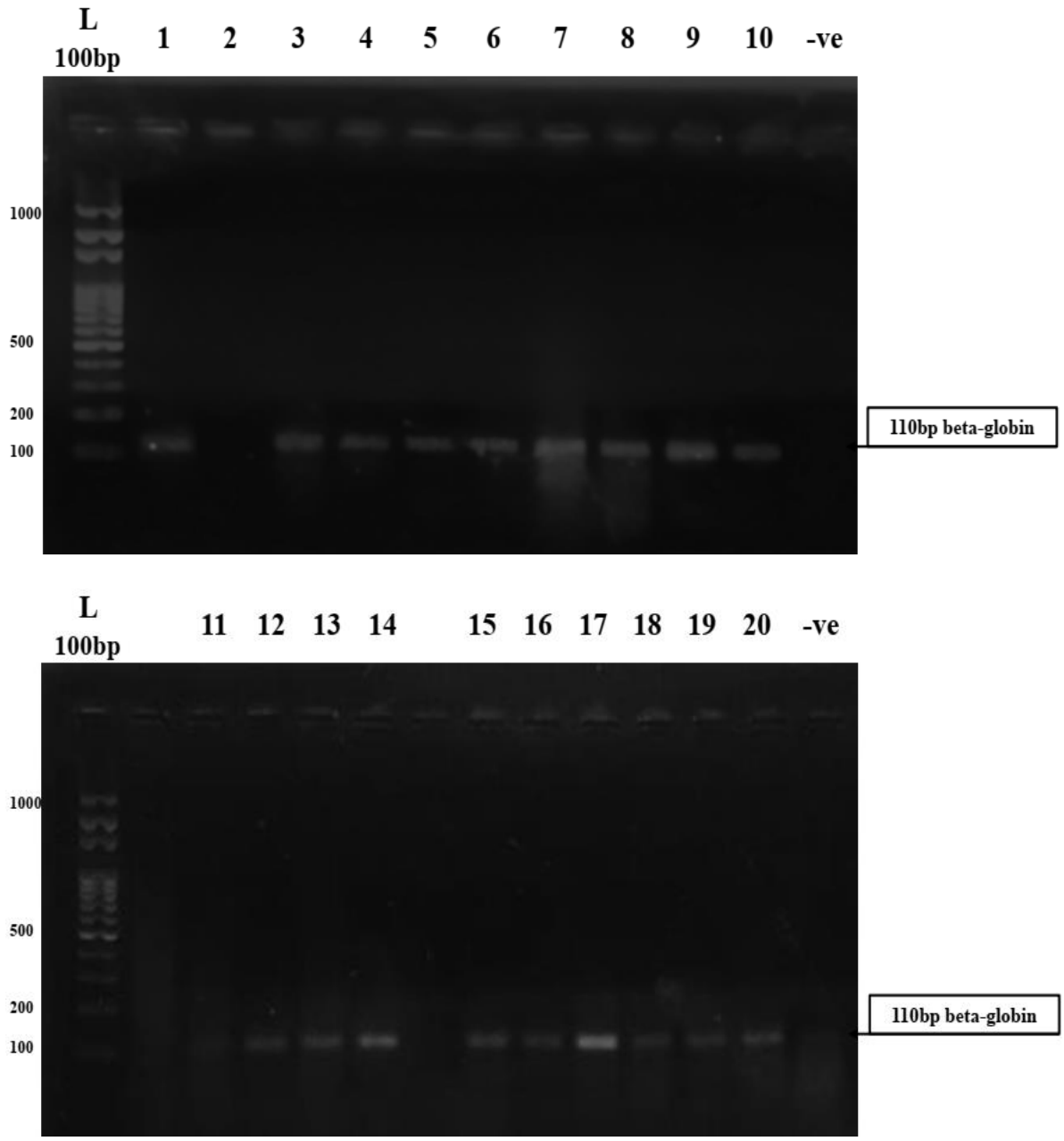


Figure 4.6 (a & b): Demonstration of validation of amplified β-globin (110bp) via 2% agarose gel electrophoresis (80V, 45min).

4.4 Optimization of HPV Consensus Primer

The PCR amplification profile of HPV consensus primer MY09/MY11 (452bp) was optimized optimized at 55°C. Figure 4.7 demonstrates the validation of optimization of MY09/MY11 (452bp) by running 2% agarose gel electrophoresis at 80V for 45min.

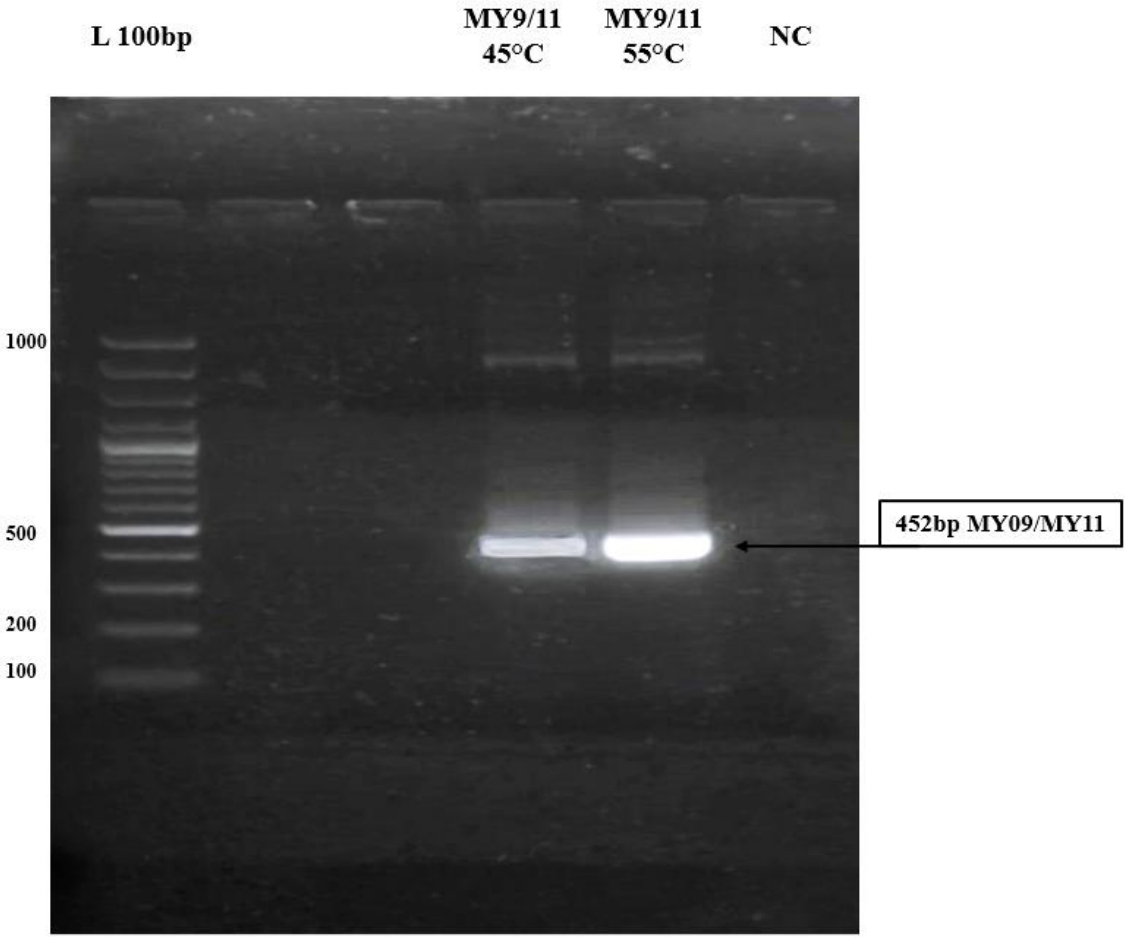


Figure 4.7: Demonstration of Validation of optimization of HPV consensus primer MY09/MY11 (452bp) via 2% agarose gel electrophoresis (80V, 45min).

4.5 Detection of HPV

After optimization, the RCC samples were tested for the confirmation of HPV. According to the results, out of 48 cases, 5 of them were HPV positive (HPV +ve) and its validation can be seen through these Figure 4.8.

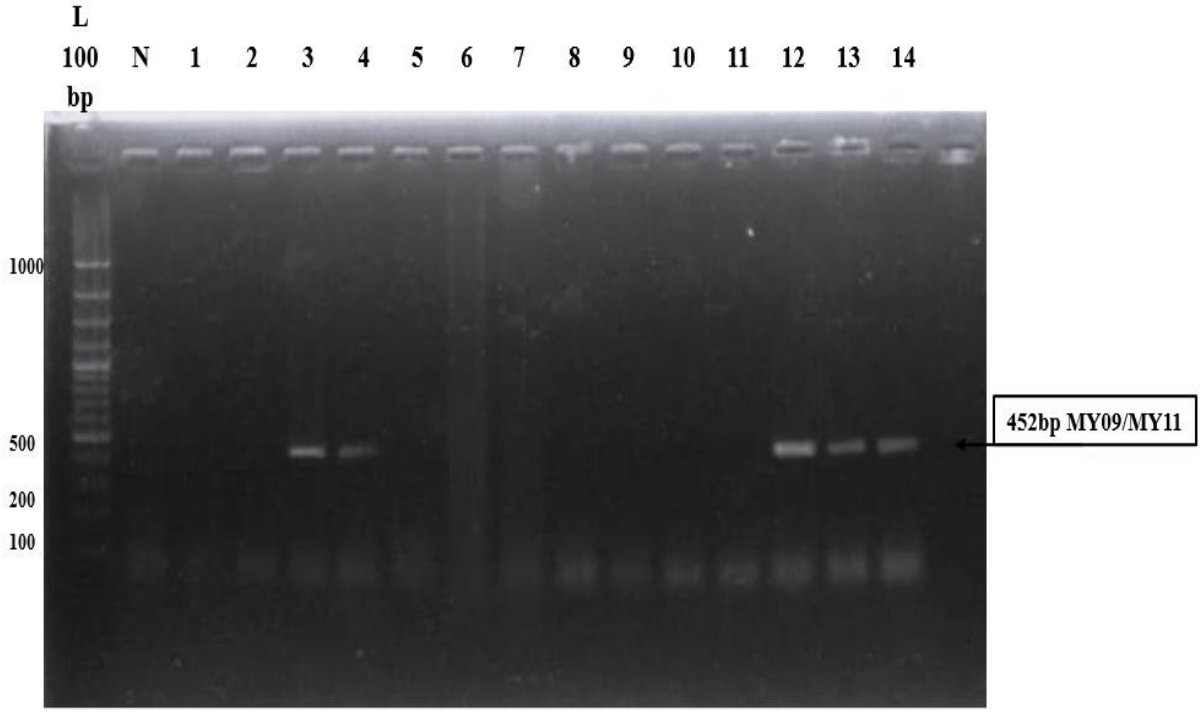


Figure 4.8: Validation of detection of HPV in RCC tissue samples by using HPV consensus primer MY09/MY11 (452bp) via 2% agarose gel electrophoresis (80V, 45min).

4.6 Characteristics of HPV Positive Renal Cell Carcinoma Patients

We categorized HPV +ve and HPV –ve RCC patients based on their characteristics like gender, marital status, education, lifestyle, smoking, and co-morbidities (Figure 4.9). According to the results, out of 48 RCC patients, 26 males were HPV -ve and 4 were HPV +ve while 17 females were HPV -ve and only one was HPV +ve which indicated that the rate of HPV +ve RCC was higher in males as compared to females. Among 5 HPV +ve RCC patients, marital status of 4 was married and only one was unmarried, 2 of these HPV positive patients were educated while 3 were uneducated. Out of 5 HPV +ve RCC patients, the lifestyle of 3 patients was less active whereas 2 of them had active lifestyle. Among 5 HPV +ve RCC patients, 4 were non-smokers and only one was smoker, and only three of them had co-morbidities.

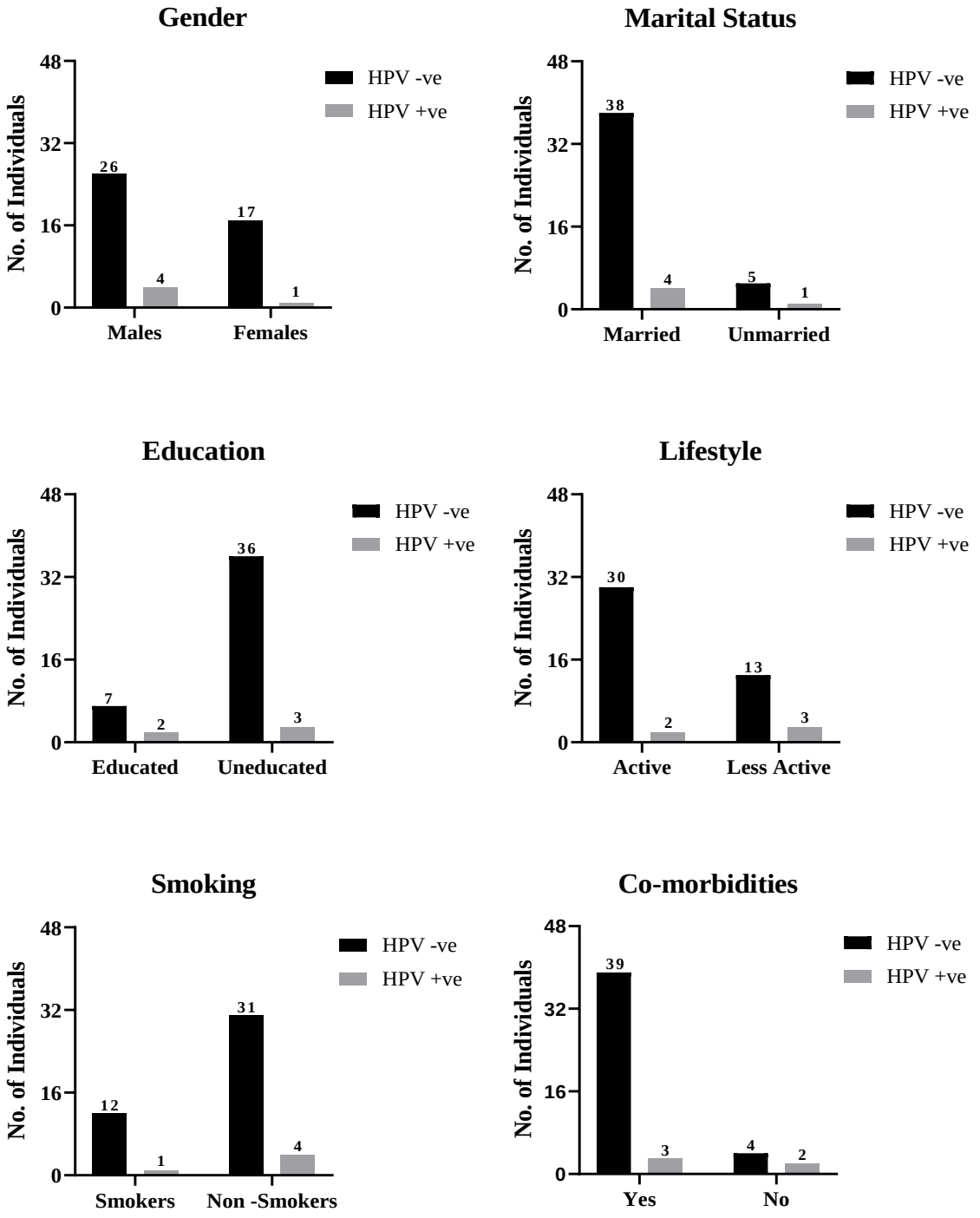


Figure 4.9: Characteristical comparison of HPV -ve and HPV +ve RCC patients.

The histopathological characteristics of HPV +ve RCC patients showed that all the patients had clear cell renal cell carcinoma. And most of them are diagnosed at Stage III. Whereas Grade 2 and Grade 3 were equally observed in HPV +ve renal cell carcinoma patients (Table 4.6).

Table 4.6: Histopathological characteristics of HPV +ve RCC patients.

Characteristics		**HPV +ve Patients (*N*=5)**
Histological Types	Clear Cell	5
	Papillary	0
	Chromophobe	0
Stages	Stage I	1
	Stage II	1
	Stage III	3
	Stage IV	0
Grades	G1	1
	G2	2
	G3	2
	G4	0

N stands for the number of HPV-positive renal cell carcinoma cases**.**

DISCUSSION

HPV is a frequent sexually transmitted disease that affects both males and females. Initially, it was known for its well-understood correlation with cervical cancer and anogenital warts, but emerging results demonstrate a link amid HPV and other malignancies including RCC. Interest in examining the part that HPV plays in the etiology and progression of renal cell cancer has increased in light of recent research that suggests a probable link between HPV infection and the onset of RCC (Salehipoor et al., 2012).

RCC accounts for a 3.2% of cancer incidences in Pakistan, placing a substantial burden on the nation's health system. However, the precise connection between HPV and RCC cases in Pakistan is still largely unknown, highlighting the need for additional research to comprehend the potential effects of HPV in relation to RCC in the nation (Mahmood et al., 2021).

RCC accounts for 85% of instances of adult kidney cancer, making it the most common type (Ljungberg et al., 2022). Studies reveal that developed nations have a higher incidence rate of renal malignancies than under-developed nations. Additionally, it is widely prevalent urological malignancy in Asia and Pakistan (Akbari et al., 2008; Chow et al., 2010). Consequently, this study was designed for investigating the clinical and histopathological characteristics of RCC malignancies in a Pakistani community. The foremost risk factors for RCC involve smoking, diabetes, hypertension and being vulnerable to occupational carcinogens etc. (Benichou et al., 1998; Moore et al., 2005).

The incidence rate of RCC in the current study was greater in males (63%) than in females (37%), this ratio of 2:1 has frequently been documented in other populations e.g., in Iran the same ratio of 2:1 of incidence rate of RCC was observed (Akbari et al., 2008; Mirzaei et al., 2015; Wong et al., 2017). The gender discrepancy in RCC incidential rate may be due to

hormonal factors. Androgen levels are higher in men, which may encourage the development of RCC cells and raise risk. Contrarily, estrogen levels are higher in women, and because estrogen has anti-proliferative and anti-inflammatory qualities, it may act as a barrier against the growth of RCC. The gender discrepancy in RCC incidence that has been reported may potentially be influenced by other factors including lifestyle, genetics, and exposure to risk factors (Laskar et al., 2019).

According to the laterality incidental data of current study, 54% of cases with RCC were reported in the right kidney, compared to 46% of cases in the left kidney. The finding shows consistency with previous study where (Humera et al., 2015) reported 60% of right-side RCC cases and 40% of left-side RCC cases. Similarly, current result shows consistency with another study where (Eggener et al., 2004) resulted 55% of right-side RCC cases and 45% of left-side RCC cases. Nonetheless, (Latif et al., 2011) reported that the left kidney was more evident than the right. Since there was no emphasis on laterality in any other investigations, this finding may be coincidental.

This study showed that approximately 43% of the RCC cases fell under a younger age group of 35-50 years indicating concordance with (Humera et al., 2015) reported 16.66% of RCC instances falling under the age group of 31-40 years. (Mohsin et al., 2012; Taccoen et al., 2007) both reported 9% and 7.5%, respectively, of RCC cases under the age of 40. The lower age group (35-50) necessitates the study of VHL alterations to distinguish the hereditary type from sporadic occurrences, while the adult age group may fall into the sporadic group. The higher number of instances in the younger age groups reported in the data may be attributable to a lack of awareness about the actual age since, majority of the cases were from lower socioeconomic and illiterate backgrounds (Palumbo et al., 2020).

Numerous previous studies have confirmed the substantial correlation between a number of variables, including marital status, education, lifestyle, residential geography, smoking, and hypertension with RCC cases. The lifestyle and environmental factors shared with the family unit may contribute to the greater frequency of RCC in married people (Padala et al., 2020; Zhang & Ro, 2015). Similarly, it has been established that living in an urban area and smoking are related to RCC occurrence. The findings that hypertension can be the leading cause for developing renal cancer also lend support to the reported relationship between hypertension and RCC (Padala et al., 2020).

The ccRCC subtype was predominantly observed in 81% RCC cases, concurring with (Humera et al., 2015) detected 70% cases as ccRCC. (Latif et al., 2011) documented 73.2% of ccRCC, (Prasad et al., 2006) documented 70% of ccRCC, (Moatasim, 2011) documented 69.2%, and (Mohsin et al., 2012) documented 68.5% of ccRCC. The significantly lower incidence rate of 7% and 6% were observed in pRCC and chrRCC subtypes respectively, showing consistency with (Humera et al., 2015) resulted that 6.6% of cases were identified as pRCC. (Prasad et al., 2006) resulted 10-15% of pRCC cases, (Ross et al., 2012) resulted 11–20% pRCC, (Latif et al., 2011) resulted 14.6% pRCC, and (Mohsin et al., 2012) resulted 9% pRCC cases. The higher incidence rate of ccRCC can be attributed to its link to common risk factors like smoking, obesity, hypertension, genetic predisposition (such as von Hippel-Lindau disease), chronic kidney disease, and specific occupational exposures. Genetic alterations in the von Hippel-Lindau (VHL) gene, in particular, are critical for the development of ccRCC because they can cause cellular process dysregulation and result in renal cell carcinoma. These elements may act together to foster the growth of tumors, increasing the higher probability of ccRCC (Muglia & Prando, 2015; Qi et al., 2021).

The results of this study showed a higher percentage of well-differentiated tumors, including Grade 2 (38%), and Grade 1 (31%) as compared to poorly differentiated Grade 3 (25%). (Kato et al., 2004) investigated 18 RCC cases and reported the Grade 2 (44.4%), Grade 1 (38%), and Grade 3 (16%) in RCC cases in Japan. (Hashmi et al., 2014) studied 64 cases and reported Grade 2 (60%) preceding by Grade 3 (36%) and Grade 4 (4%). The disparity in these results is most likely due to varying sample sizes.

In this study, out of 48 RCC patients, HPV was detected only in 5 (10.4%) patients which showed consistency to the results of a small case-control investigation including 49 RCC cases resulted that the level of HPV prevalence was relatively 14.3% (Salehipoor et al., 2012).

5.1 Conclusion

This study revealed a understanding of the clinical and histological aspects of RCC patients in Pakistan. The predominance of the ccRCC subtype, the higher frequency of RCC in males, and the importance of histological Grade 2 appeared in accordance with previous investigations. The existing research additionally confirmed the correlation between RCC and specific demographic variables including age, marital status, education, lifestyle, residential geography, smoking, and hypertension. Moreover, the study showed 10.4% prevalence of HPV in RCC patients indicating HPV as a contributing factor to the development of RCC in the Pakistan population.

5.2 Future Prospect

Further confirmation with increased sample size and establishment of a robust correlation between HPV and RCC can be attained through the implementation of HPV genotype-specific

testing, with particular emphasis on detecting the presence of HR-HPV genotypes, notably HPV-16, & -18.

It is necessary to do additional investigations with more extensive and varying cohorts in order to confirm and build on these findings. For example, addressing additional possible risk variables, such as familial genetics, working environments, and food choices, may help to comprehend all the causes of RCC in Pakistan.

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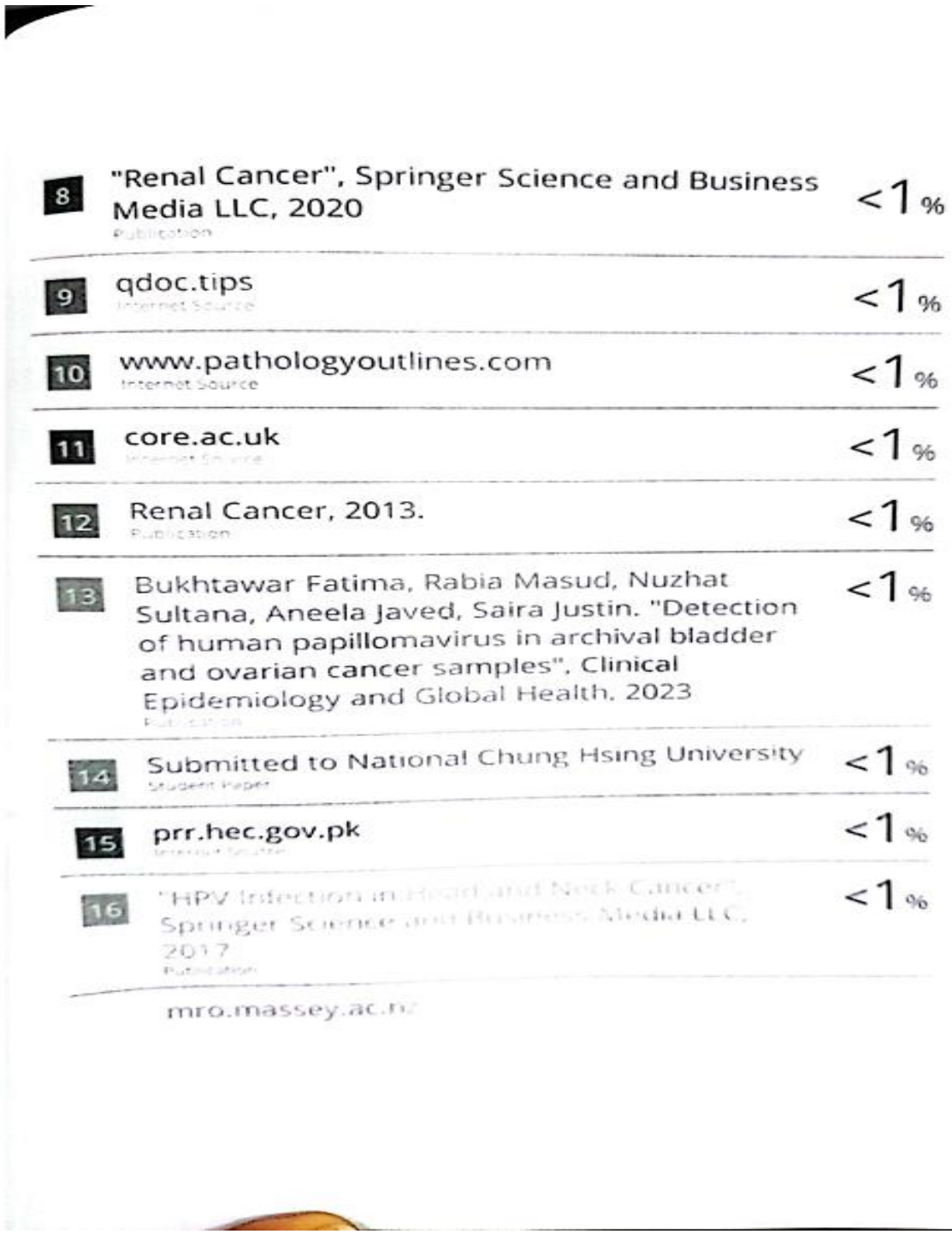

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