DETECTION OF HUMAN PAPILLOMA VIRUS USING P16^{INK4A} IN MALIGNANT TUMORS OF URINARY BLADDER AND URETHRA IN PATIENTS AT KENYATTA NATIONAL HOSPITAL

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DECLARATION

I, **DR. MICHIEKA OKIOGA MICHIEKA,** declare that this is my original work, and has not been presented for any award in any other university or institution.

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ABSTRACT

Background: Bladder cancer is ranked 7th as the most common cancer globally accounting for approximately 3.2% of all global cancers. Urethral carcinomas are rare but aggressive tumours that account for less than 1% of all genitourinary malignancies. Human Papillomavirus (HPV) is the most common viral infection of the reproductive tract and has been linked to most cancers compared to any other virus. It has a well established link with malignancies especially anogenital tract, head and neck cancers. HPV has the ability to cause malignant transformation of epithelial cells through oncogenicity of early viral proteins, whose major role is the malignant transformation through cell cycle regulation disruption. The role of P16 as a surrogate marker for HPV infection has an established use in clinical practice. Various studies employing P16 have been done to show the causal relationship and attribute HPV infection to bladder and urethral tumours with varying prevalence results ranging from 0 % to 35–52 %.

Objective: To determine the prevalence of human papilloma virus in malignant tumours of urinary bladder and urethra at Kenyatta National Hospital, using $P16^{INK4A}$immunostaining.

Materials and methods: This was a laboratory based, retrospective descriptive study using archived tissue blocks of malignant tumour presence from January 2014 to December 2016.

Results: Males were 61% while females were 39%. Age range was between 34 and 80 years with a median age of 62. Most of the cases (64%) were between the ages of 51 to 70 with a peak at 61 to 70 years. The malignant tumours for the urethral cases included 2 invasive squamous cell carcinoma and 1 non-invasive low-grade urothelial tumour. Urinary bladder samples were 95% of the cases. These were categorised as 39 (63.9%) being infiltrating and 22 (36.1%) as non-invasive. According to histological type, urothelial tumours made up the majority, 57 comprising 93.4% of cases. Other types were glandular 2 (3.4%), squamous 1 (1.6%) and neuroendocrine 1 (1.6%). For the 57 urothelial tumours, infiltrating were 35 with

low grade being 11 and high grade being 24. For the non-invasive which were 22, low grade were 13 and high grade were 9. There was one case of poorly differentiated infiltrating squamous cell carcinoma, one case of infiltrating small cell carcinoma and two cases of adenocarcinoma - poorly and well differentiated. Nine cases were positive for p16 expression and these were all urinary bladder tumours (14.1%). From these nine positive cases, eight were urothelial carcinomas with five being high grade and three low grade tumours. Five were infiltrative and four non-invasive. There was one positive case of an infiltrating poorly differentiated squamous cell carcinoma.

Conclusion: The use of P16 as a surrogate marker for HPV infection has aided in the identification of tumours attributed to HPV. In this study, the findings demonstrated the presence of HPV in 14% cases of infiltrating and non-invasive urothelial carcinoma using P16. Therefore, HPV is most likely an etiological agent of a proportion of urinary bladder cancers similar to cervical and anal cancers.

Recommendations: The routine use of molecular analysis in order to establish the actual burden of urothelial neoplasms attributable to HPV in our population.

The demonstration of HPV as a cause of bladder cancer is significant in the development of prevention strategies in our population. Similar to HPV vaccination for cervical cancer, a case for vaccination of boys can be included among other prevention strategies such as cessation of smoking, occupational and environmental exposure risk reduction.

Key words: Bladder cancer, urethral cancer, Human Papilloma Virus, $P16^{INK4A}$

LIST OF ABBREVIATIONS

CIN: Cervical Intraepithelial Neoplasia

CIS: Carcinoma in situ

DNA: Deoxyribonucleic acid

ELISA: Enzyme-Linked Immunosorbent Assay

ERC: Ethics Research Committee

H&E: Haematoxylin and Eosin

HIER: Heat induced epitope retrieval

HIV: Human Immunodeficiency Virus

HPV: Human Papilloma Virus

HR-HPV: High Risk Human Papilloma Virus

HSV 1: Herpes Simplex Virus 1

HSV 2: Herpes Simplex Virus 2

IARC: International Agency for Research on Cancer

IHC: Immunohistochemistry

ISUP: International Society of Urological Pathologists

KNH: Kenyatta National Hospital

LCR: Long Chain Region

LUT: Lower Urinary Tract

PAR: Population attributable risk

pRB: Retinoblastoma gene

SCC: Squamous cell carcinoma

SEER: Surveillance, Epidemiology and End Results

SPSS: Statistical Package for the Social Sciences

TCC: Transitional cell carcinoma

UC: Urothelial carcinoma

UBC: Urinary bladder cancer

UON: University of Nairobi

UTI: Urinary tract infection

WHO: World Health Organisation

INTRODUCTION

1.0 Introduction

According to Vignozzi *et al*. (2016), the components of the Lower Urinary Tract (LUT) are the urinary bladder and urethra in females while in males the prostate is also included. Vignozzi *et al.* (2016), adds that the role of LUT is the non-volitional storage of urine received from the upper urinary tract at low pressures and the controlled urine expulsion when it is generally tolerable. While studying some of the functional disorders affecting the LUT, Lienert *et al.* (2013), states that, over active bladder, urinary incontinence or obstructing disorders such as prostatic enlargement and stricture disease and pathologies such as carcinoma and lithiasis are the main disorders.

Vaidya *et al*. (2013), ranks bladder cancer as the 7th most common cancer globally, with approximately 260,000 and 76,000 new cases annually in men and women respectively. Cancer statistics by Siegel *et al.* (2014), demonstrate that urinary bladder cancer is more significant in males than in females with a global ratio of 3.5:1in 2014. At the same time, urinary bladder cancer accounted for roughly 3.2% of all worldwide cancers.

According to Humphrey (2012), primary urethral carcinomas are rare but aggressive tumors that account for less than 1% of all genitourinary malignancies. Due to the rarity of the tumor, there is not much data available on it. A Surveillance, Epidemiology, and End Results (SEER) study by Siegel *et al.* (2014), on approximately 10% of the total USA population reported a 4.3 and 1.5 incidence per million of urethral carcinoma in males and females respectively. Incidence increased with age, peaking at ages 75-84 years, at 32 and 9.5 per million in males and females respectively.

In their study, referred to as the RARECARE project, Stiller et al. (2013), revealed an age-adjusted incidence of 1.6 and 0.6 cases per million of urethral carcinoma in males and females

respectively. They dealt with data spanning from 1995-2002 from 32% of all the 27 member states from the European Union.

The RARECARE project agreed with the findings of the SEER study previously conducted and found out that the incidence increased with age, with the peak being experienced in patients whose age was 75 years and above (Stiller *et al.*, 2013).

In Kenya, data from the Nairobi Cancer Registry (2000-2002) indicated a prevalence of 2% for bladder cancer and 0.1% of penile cancer in males, while in females, bladder cancer comprised 0.8% of all reported cancers (Korir *et al.*, 2017).

1.1 Human Papilloma Virus

Stanley (2012) describes the Human papillomavirus (HPV) as the most common viral infection belonging to the papillomavirus family affecting the reproductive tract in most sexually active men and women either repeatedly or at some stage in their lives. Cubie (2013) studied exclusively the biology of this virus and linked it with well-established anogenital tract cancers such as the cervical, vaginal, vulva, penile, anal and the head and neck cancers. A study on the evolution of the papillomaviridae by Van Doorslaer (2013) reveals that HPV has more than 150 sub-types, categorized into either high or low risk depending on the ability to induce malignant transformation. He further states that, the virus is associated with the development of more cancers in humans than any other virus. In addition, less than 10% infections related to HPV clear up within a few months after acquisition without any medication while 90% clear in 2 years. Only a small proportion of certain HPV infections persist and progress to cancer with cervical cancer being the most common HPV-related disease.

Globally, HPV 16 and 18 contribute to approximately 70% of the total cervical cancer cases. However, vaccines that prevent such infections are available with a potential to reduce the incidence of cervical and other anogenital cancers (Schiffman *et al.*, 2013).

Noguera (2015) identifies tobacco use and occupational or environmental exposure as risk factors responsible for the development of LUT tumors. Thus, prevention measures limit themselves to these risk factors making it hard for the prevention of bladder cancer that is more aggressive, costly and takes a long duration to treat. In addition, insufficient quality markers for prognosis and therapeutic response further hinder the treatment of bladder cancer. Global cancer statistics by Torre *et al.* (2015), estimates an approximate of 12% of global cancer cases as viral infections and states that the discovery of HPV as a cause of bladder cancer is significant in the development of prevention and therapy strategies.

While most studies demonstrate an unclear HPV role in bladder cancer, several studies that have been published reveal a varying association of HPV to bladder cancer; a result that such studies attribute to patients' diversity, low case numbers, and analysis that is restricted to a select set of HPV types. In addition, probable contamination during HPV analysis has also led to an unclear HPV role in bladder cancer as this may be a cause of false positive results that may happen during the cutting of formalin fixed paraffin-embedded tissue, sample preparation or DNA analysis process (Malats *et al*., 2015).

LITERATURE REVIEW

2.1 Epidemiology of Urinary Bladder and Urethral Malignancies and Human Papilloma Virus

According to GLOBOCAN (2018) urinary bladder cancer incidence cases are 549,393 with mortality being 199,992 of these cases. For the male population, the incidence is 330,380 (4.5%) with a mortality of 123,051 (2.6%). For females, the incidence is 99,413 (1.5%) with a mortality of 42,033 (1.2%). In Africa, male prevalence is 17,685 (4.9%), mortality 9,362 (3.4%). Female prevalence 6,752 (1.4%), mortality 3,906 (1.2%). For both sexes, the prevalence is 24,437 (2.9%), mortality 13,268 (2.2%).

In Kenya, male prevalence is 231 (1.3%) with a mortality of 133 (1.0%) while female prevalence is 117 (0.5%) with a mortality of 63 (0.4%). Both sexes prevalence is 348 (0.8%), mortality 196 (0.7%). Primary urethral carcinomas are rare aggressive tumours accounting for less than 1% of all genitourinary malignancies. Due to the rarity of the tumour, there is not much data available on it (GLOBOCAN, 2018).

Global estimates of HPV prevalence as documented from statistics stands at 11.7% with sub-Saharan Africa averaging at a prevalence of 24.0%. In Eastern Africa within which Kenya falls it is 33.6%. In Kenya, the prevalence of women with HPV in a study done by De Vuyst in Mombasa in 2010 was 40.3 %. In a similar study done in Nairobi by De Vuyst in 2003, the prevalence stood at 38.8%. In 2013, Maranga reported a prevalence of 41.6% in Nairobi. Ng'ayo, in 2008, found the prevalence to be 57.6% among men working in the fishing industry in Kenya. Smith, in 2010, found it to be 51.1% among Kenyan men screened who participated in male circumcision (Orock, 2015).

2.2 Anatomy of Lower Urinary Tract

The LUT comprises the urinary bladder and urethra in women with the addition of the prostate in men.

2.2.1 Urinary Bladder

The urinary bladder is located in the anterior pelvis of adults. It is enveloped by extraperitoneal fat and connective tissue and separated by space of Retzius (an anterior pre-vesical space) from the pubic symphysis. The bladder dome is covered by peritoneum, and the bladder neck is attached to adjacent structures by reflections of the pelvic fascia and by true ligaments of the pelvis. The pelvic diaphragm in females and the prostate in males give it inferior support. It is composed of the bladder neck which has a muscular wall organized into 3 layers. The trigone which forms the bladder floor and the ureters insert at this point. Vascular supply is derived from the internal iliac (hypogastric) arteries while venous drainage is through the internal iliac vein. The lymphatic drainage of the bladder is into the obturator, external iliac, internal iliac (hypogastric), and common iliac lymph nodes. Innervation is supplied by branches of the vesical and prostatic plexuses (Mahadevan, 2016).

2.2.2 Urethra

The urethra is a fibromuscular tube that conducts urine from the bladder (and semen from the ductus deferens) to the exterior. It originates at the neck of the bladder, passes through the pelvic and urogenital diaphragms, and ends at the external urethral orifice.

The female urethra, which is about 4cm in length, is fused with the anterior wall of the vagina. It ends between the clitoris and the vagina. The male urethra, with a length of about 20 cm, has three parts: prostatic, membranous, and spongy (Sulaiman *et al*., 2018).

2.3 Microscopic Anatomy

2.3.1 Bladder

The bladder wall is most organized at the bladder neck, with 3 layers-the inner longitudinal muscular layer, middle circumferential muscular layer and outer longitudinal muscular layer The trigone has a mucosal layer deep into which are 2 muscular layers (Sulaiman *et al*., 2018).

2.3.2 Urothelium or Bladder Mucosa

The bladder mucosa is transitional epithelium and is loosely connected to the muscular bladder wall by the lamina propria that serves as a connective tissue layer. The bladder submucosa or lamina propria is rich in microvasculature and overlies the detrusor muscle. At the trigone, the epithelium is more densely adherent to the underlying muscle (Sulaiman *et al*., 2018).

2.4 Histology

2.4.1 Urinary Bladder

Bladder layers are mucosa (urothelium, lamina propria, discontinuous muscularis mucosa), muscularis propria, adventitia, serosa/peritoneum at the dome. No sub-mucosa is present.

2.4.2 Microscopic (Histologic) Description

2.4.2.1 Urothelium

The urothelium is intermediate between non-keratinizing squamous and pseudostratified columnar epithelium and thus was originally referred to as the transitional epithelium. Its main feature is that it is 5-7 cell layers thick in the contracted bladder and 2-3 layers thick in the distended bladder.

It lines the renal pelvis, ureters, bladder, most of the urethra but not the distal urethra. The superficial urothelium (umbrella cell layer) is a single layer of umbrella cells, which are large and elliptical with abundant eosinophilic cytoplasm.

Intermediate urothelial cells are cuboidal to low columnar with well defined borders and amphophilic cytoplasm. Basal urothelial cells are more cylindrical and can be flat when the bladder wall is stretched (Paner *et al*., 2017).

2.4.2.2 Lamina propria

It is located between the mucosal basement membrane and the muscularis propria and is thinner at the trigone and the bladder neck. It contains loose to dense connective tissue and variably sized blood vessels (Paner *et al.,* 2017).

2.4.2.3 Muscularis propria

It consists of inner longitudinal, circular and outer longitudinal layers of thick muscle bundles. It may also contain adipose tissue between muscle fascicles. Muscle layers are distinct only near the bladder neck. The longitudinal and circular layers mix freely without definite orientation (Paner *et al.,* 2017).

2.4.2.4 von Brunn's nests (proliferative cystitis)

Reactive proliferative cystitis are present in 85-95% of bladders with their frequency increasing with age. They are more common at the trigone. They are nests of cytologically benign urothelium in lamina propria. The nests have regular spacing and extend to same horizontal level at base of proliferation (Paner *et al.,* 2017).

2.4.3 Urethra

The urethra is a fibromuscular tube lined with urothelium, columnar epithelium and non-keratinizing squamous epithelium. The sub-epithelium comprises of loose fibroelastic tissue, glands and abundant vessels. Muscle layers include smooth muscle and exterior skeletal muscle (Paner *et al.,* 2017).

2.5 Etiology of LUT Neoplasms

2.5.1 Etiology of Urinary Bladder Cancer

Bladder cancer can be caused by the following: inhalation of certain agents such as cigarette smoke**,** cooking fume hood, industrial carcinogens including aromatic amines used for chemicals and components in industrial use, volatiles of coal tar, diesel and gasoline exhausts**;** consumption of drugs including cyclophosphamide, cloronaphazine, phenacetin, nitrosamines, herbal remedies; skin contact by chlorinated water; dietary factors; chemical contaminants such as arsenic; endogenous carcinogens; infections including HPV and Schistosomiasis and also hereditary factors (Alexander *et al.*, 2012).

According to Chung (2013), HPV detection is in four out of five transitional cell carcinomas arising in renal transplant recipients. The study also suggested that HPV 16 might be the causal pathogen in bladder cancer development. However, Syrjaenen (2013) studied HPV prevalence in oesophageal squamous cell carcinoma and positive HPV antigen results in 47 out of 78 samples but negative for HPV DNA. A study on HPV in the bladder of Tunisian patients through morphological and PCR examination detects no HPV infection in any case of bladder carcinoma. This makes HPV infection as a risk factor contributing to the development of bladder cancer, an open debate (Alexander *et al.*, 2012).

2.5.2 Etiology of Urethral Cancer

The cause of urethral cancer is unknown. History of bladder cancer increases urethral cancer risk. Previous studies documented infection and prolonged irritation as probable causes of urethral cancer. For instance, 37% of males suffering from urethral cancer as studied by Kaplan *et al.* (2006), were found to have suffered from venereal diseases. Additionally, O'Brien *et al.* (2011), found a strong relationship between urethral cancer and STD's history on 44% of urethral cancer patients selected from British research centres. Other studies have also

documented evidence of a strong relationship between urethral cancer and HPV. For example, Wiener *et al*. (1994), revealed the existence of HPV DNA in 29% of urethral cancer cases.

Another cause of urethral cancer is chronic inflammation. In their study, Jungwirth *et al.* (2012), found that 88% of urethral cancer male patients had previously suffered from stricture while Bunker *et al*. (2015), found a strong correlation in 16% of urethral cancer in Indian patients. Other instances such as in Burger *et al.* (2013), associated arsenic ingestion as a risk factor for primary urethral cancer.

2.5.3 Pathophysiology

Given the low incidence of urethral cancer, specific pathophysiologic considerations are unknown. However, chronic inflammation, infection or irritation of the urethra is precedes urethral cancer development as they lead to a rapid turnover of the mucosal cells in the urethra influencing dysplasia and neoplasia development. In addition, they may also hinder the natural repair mechanisms of the DNA in the urethral mucosal cells. The tumor usually invades deeply and metastasizes to adjacent structures (Eynard *et al*., 2013).

2.6 Types of Urinary Bladder Tumors

According to Lopez-Beltran *et al*. (2016), the types of urinary bladder tumours are transitional cell bladder cancer/Urothelial carcinoma, squamous cell carcinoma, adenocarcinoma, small cell carcinoma and sarcoma.

2.7 Classification of Urinary Tract Tumors

Table 1 gives the classification of urinary tract tumours as classified in WHO / ISUP Classification of Tumors of Urinary Tract (2016)

Table 1: Classification of urinary tract tumors

Urothelial tumours	**Mesenchymal tumours**
Infiltrating urothelial carcinoma ● with squamous differentiation ● with glandular differentiation ● with trophoblastic differentiation ● Nested ● Microcystic ● Micropapillary ● Lymphoepithelioma-like ● Lymphoma-like ● Plasmacytoid ● Sarcomatoid ● Giant cell ● Undifferentiated Non-invasive urothelial neoplasias ● Urothelial carcinoma in situ ● Non-invasive papillary urothelial carcinoma, high grade ● Non-invasive papillary urothelial carcinoma, low grade ● Non-invasive papillary urothelial neoplasm of low malignant potential ● Inverted urothelial papilloma ● Urothelial papilloma	● Rhabdomyosarcoma ● Leiomyosarcoma ● Angiosarcoma ● Osteosarcoma ● Malignant fibrous histiocytoma ● Leiomyoma ● Haemangioma ● Other
Squamous neoplasms ● Squamous cell carcinoma ● Verrucous carcinoma ● Squamous cell papilloma	**Melanocytic tumours** ● Malignant melanoma ● Nevus

Glandular neoplasms Adenocarcinoma ● Enteric ● Clear cell ● Signet-ring cell ● Mucinous Villous adenoma	**Haematopoietic and lymphoid tumours** ● Lymphoma ● Plasmacytoma
Neuroendocrine tumours ● Carcinoid ● Small cell carcinoma ● Paraganglioma	**Miscellaneous tumours** ● Carcinoma of Skene, Cowper and Littre glands ● Metastatic tumours and tumours extending from other organs

Source: WHO/IUSP (2016)

2.7.1 Types of Urethral Tumors

Stephens *et al.* (2009), cites squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma, melanoma and sarcoma as the only types of urethral tumours.

The authors further add that transitional cell carcinoma develops in the surface cells of the urethra, adenocarcinoma in glands located adjacent to the urethra, melanoma though extremely rare develops in pigment-producing skin cells, while sarcoma also extremely rare grows in blood vessels, smooth muscle, and connective tissue.

2.8 Human Papilloma Virus

Khode *et al.* (2014), describes papillomaviruses as small, non-enveloped, highly epitheliotropic, double-stranded DNA viruses that infect mucosal and cutaneous epithelia. Close to half of the more than 100 types of HPVs identified, infect the genital tract (Torre *et al.* 2015), with many types being found in cervical cancers. Others are rarely found or not at all found in a large number of cancers resulting to low risk and high-risk classification of HPVs.

The capability of viruses or their genomic constructs to stimulate strongly homologous and heterologous promoters in cells that express epithelial markers such as certain keratin genes is referred to as epithelial specificity. Genital HPVs' epithelial specificity is controlled by epithelial specific transcription factors that attach to explicit locations in the long chain region (Watts *et al*. 2014).

Based on the relative occurrence of viral DNA in certain cancers, HPV types 16 and 18 are considered to be high cancer risk; about 15 other less common types are also considered high risk. Viral early proteins E6 and E7 are synthesized in cancer tissue; these are HPV transforming proteins, able to complex with Rb and p53 and other cellular proteins.

2.8.1 Epidemiology of Human Papilloma Virus Associated Neoplasms

An analysis by Workowski (2015) on approximately 660 million people globally with HPV infection revealed a peak incidence of HPV infections occurring among adolescents and young adults below 25 years.

In addition, over 90% and 80% of cases of cervical cancer and anal cancer respectively are associated with HPV infections. With a variety of HPV types causing genital infections, HPV 16 or 18 are most frequently found in cervical carcinomas, even though some cancers contain DNA from other types, such as HPV-31. More than 70% of all cervical cancers can be attributed to HPV-16 and 18, where type 16 is the most common (Tommasino 2014).

Anal cancer is associated with high risk HPV infection. Patients who are immunocompromised and homosexual men are especially at risk. Multiple types of HPV have been found in the anal canals of HIV-infected homosexual men.

Oropharyngeal cancers, a subset of head and neck squamous cell carcinomas, are also linked to HPV infections, especially by type 16. For instance, close to 25% of oral and 35% throat cancers respectively have an association with HPV. While assessing the epidemiology and

clinical aspects of HPV in head and neck cancers, Deneka *et al.*(2018), found that oral cavities of HIV-positive and negative persons contained numerous types of HPV.

2.8.2 Cancer in Humans Attributed to HPV

Table 2 lists HPV associated cancers as discussed in WHO/IARC HPV evaluation of carcinogenic risk (2007) and malignancies in various anatomical sites.

Table 2: Table showing HPV associated cancers

Cervical Vulva Vagina Penis Anus Oral cavity Oropharynx and tonsil Oesophagus Larynx	Skin Nose and nasal sinuses Colon and rectum Lung Ovaries Prostate Breast
Urinary bladder and Urethra	

Source: Adapted from WHO/IARC: Human Papilloma Virus: Monographs on the evaluation of carcinogenic risks to humans (2007)

2.8.3 Mechanism of HPV Associated Carcinogenesis

Persistent infection with high-risk HPV, through the expression of E5, E6, and E7 alters the pathways related to cellular transformation and immune escape and is a critical step towards the development of malignancy. Despite this, it is not adequate for progression to cancer as persistent infections are only observed in approximately 10% of individuals. This is due to the fact that the immune system clears a majority of HPV infections in a relatively short duration (Tommasino, 2014).

For example, for cervical cancer to progress to invasive characteristics in most patients, a long duration of time is normally required for multiple steps to take place for the development of low or high-grade cervical intraepithelial neoplasia (CIN) after a latency period.

However, the clearance of persistent infection as well as the worsening of these pre-cancer injuries is sometimes detected. Facts that suggest existence of other viral risk factors such as oncogenic events report that a large number of women infected at no point in their lifetime develop cancer (Giesecke, 2017).

By the induction of centrosome abnormalities, DNA damage and increased frequency of foreign DNA integration in the host genome, high risk E6 and E7 expression can induce genomic instability since E6 and E7 act in cooperation to induce abnormal centrosomes accumulation, including chromosomal mis-segregation and the development of aneuploidy. In addition, E6 and E7 also cooperate to override mitotic checkpoints leading to aneuploidy, a phenomenon that might be critical for viral replication in addition to genomic instability (Akagi *et al.* 2014).

Despite its nuclear location as an episome through the initial infection, the integration of HPV into the host genome takes place in the progression to high grade dysplasia. Thus it is thought to be related with the progression from low to high grade intraepithelial neoplasia (Burk *et al.* 2013). Double-strand breaks of DNA, thought to be induced by the expression of E6 and E7 are necessary for the virus integration into the genome host. Though HPV is contained in most lesions, high grade lesions have traces of episomal and integrated HPV combined while the pure episomal form is rarely observed in the invasive stages (Galloway *et al*. 2015).

According to Galloway *et al.* (2015), E2 expression is frequently disrupted by HPV integration, leading to increased expression of E6 and E7 that results from the subsequent inability to suppress the expression of viral oncogenes.

This is an implication that that HPV integration has the capacity to stabilize the transcription of oncogene in addition to affording growth and survival advantages by altering important cellular genes and changes in global promoter methylation and transcription.

A study by Ojesina *et al.* (2014), reported that basic cellular genes, for instance RAD51B, NR4A2, and TP63 are deregulated by HPV integration. Hu *et al.* (2015), used whole-genome sequencing and high-throughput viral integration detection to identify HPV integration hotspots and resolved that microhomology-mediated DNA-repair pathways could enhance these integration events. They further added that genetic alterations as a result of genomic instability and integration of HPV DNA into the genome host, epigenetic modifications may operate in the progression of the disease.

As discussed by Tommasino (2014), most epigenetic modifications take place in the course of different stages in both the HPV and host cellular genomes. Despite the fact that the role of viral methylation in the infected cell on malignant transformation is unclear, modified HPV methylation in L1 and L2 regions is commonly observed. Johannsen *et al*. (2013), speculated that the methylation of the four E2-binding sites (E2BSs) contributes to the deregulation of E6 and E7 expression during the progression of the disease.

2.9 HPV Prevalence in Lower Urinary Tract Tumors

Kim *et al.* (2014), did a study to assess the potential association between HPV infection and the squamous cell component of urothelial carcinoma (UC) of the bladder and to validate overexpression of p16 as a surrogate marker for HPV infection in cancers among Koreans, using HPV-DNA chip and p16 expression by use of immunohistochemistry. The experimental group comprised of patients possessing squamous differentiation of UC of the bladder while the control was composed of patients with squamous metaplasia of the bladder.

The study found out a 2-fold higher HPV DNA detection rates in experimental as compared to control, that is 17.1% versus 8.3% respectively. The study also detected overexpression of p16 in 45.7% of the experimental group as compared to 8.3% of the control group. In addition, both positivity of HPV and overexpression of p16 were detected in 8.8% of the experimental group but none in the control group. On assessing the risk factors, HPV cases that were positive were 2-fold higher for smokers as compared to non-smokers, that is, 66.7% and 31.0% for smokers and non-smokers respectively. The study thus concluded that infection of HPV may be related with UC of the bladder with squamous differentiation, especially in non-smokers.

Chapman-Fredricks *et al.* (2013), reported that 37% of primary SCCs of urinary bladder demonstrated diffuse p16 immunoreactivity independent of sex or tumour differentiation. The study further observed an overexpression of p16 in most of the primary bladder SCCs, leading them to question whether this overexpression of p16 could be as a result of HPV-dependent mechanisms. From their previous observation where a subset of primary bladder SCCs overexpressed p16 by immunohistochemistry, they hypothesized that HR-HPV DNA might be present in this cancer subtype. Therefore, they set out to seek whether HR-HPV DNA could be present within cells of primary bladder SCC that were p16 positive by using in situ hybridization and a third-generation signal amplification Invader assay, followed by PCR amplification and sequencing for confirmation. The invader technology revealed that 21% of their cases had HR-HPV DNA within tumour tissue.

While determining the association between HPV and transitional cell carcinoma (TCC), Barghi *et al.* (2012), used polymerase chain reaction on 59 bladder tissue specimens collected from TCC patients and compared it with 20 bladder samples of cases with non-neoplastic disorders. The study detected HPV DNA in 35.6% of the 59 tissue specimens compared to only 5% in the control group. In addition, it was found out that HPV 18 was the most common virus type

at 81% and thus it was concluded that HPV might play a causative role in transitional cell carcinoma of bladder in Iran.

In an experiment to ascertain the degree of relationship between bladder cancer and HPV infection, Jimenez-Pacheco *et al.* (2012), conducted a meta-analysis using experimental and control groups through analysing the pooled effect of all the studies and techniques used. By use of chi-square tests and pooled odds ratio (OR), the study pointed out to a significant relationship between HPV and bladder cancer. The study also concluded that a moderate relationship existed between viral infection and bladder tumours as evidenced by the pooled OR values.

The prevalence of HPV in bladder cancer as studied by Li *et al.* (2011), by use of pooled data that took consideration of heterogeneous related parameters-such as region, histological type, HPV DNA specimen, publication calendar period, and detection method; revealed the following results: 16.88% HPV prevalence at 95% confidence interval of which 15.82% were high risk that varied by region. The study also revealed statistically significant increased risk of bladder cancer influenced by HPV type, study region, HPV DNA specimen, and detection method. The conclusion from this study was that high-risk HPV types infection may be critical in bladder carcinogenesis.

The role of HPV types 16, 18 and 52 in recurrent cystitis and urinary bladder cancer among 60 Egyptian patients as studied in Badawi *et al.* (2008), estimated their prevalence. The study was conducted on group I patients with histopathologically and clinically evident bladder cancer, group II patients with cystitis and group Ⅲ patients with bladder cancer with cystitis and a control group for serologic testing. The experiment detected 30% and 10% of HPV-16 and -18 in BTB with higher rates that are significant in bladder cancer than cystitis cases (44.4% and 11.11% respectively), with significant association with schistosomal infection (78.6% and

25%, respectively) and recurrence (48%, HPV-16). The study also revealed a significant relationship of TCC with HPV-16 and also showed that multiple HPV-16, -18 and -52 were significantly higher than single types with 79.2% and 20.8% respectively. The study further confirmed a substantial relationship between HPV-16, -18 and -52 with bladder cancer and suggested viral synergistic action in bladder carcinogenesis.

Alexander *et al.* (2014), enrolled 85 patients in his experiment to detect HPV infection, *p21* oncogene, DNA content of urothelial cells in different bladder lesions with and without schistosomiasis and correlated them with histopathological grade and stage. In addition, the study also used 10 healthy individuals as a control. By using in situ hybridization and immunohistochemical technique in formalin-fixed, paraffin-embedded tissues, the experiment revealed that HPV DNA 6/11 and 16/18 expression was increased from cases of schistosomal cystitis with dysplasia to TCC with schistosomiasis compared to TCC and SQCC. The conclusion in this study is that both HPV infection and p21 gene abnormalities might be contributory to bilharzial bladder carcinogenesis.

The sensitivity of an in situ hybridization system was investigated to detect HPV infection in transitional cell bladder by De Gaetani *et al.* (1999), and also examined the relationship between HPV tumour infection and the presence of serum anti-HPV antibodies detected by in situ hybridisation and enzyme linked immunosorbent assay (ELISA) respectively.

The study also related viral infection presence to grade, stage and follow up in bladder cancer cases. In situ hybridisation technique demonstrated the presence of HPV DNA in 17/43 bladder cancer cases while 15/43 had negative HPV DNA. In 14/43 cases, HPV types were either 16/18 or 31/33/35, all carrying high oncogenic risk. The correlation between stage and grade of the tumour with HPV detection demonstrated that ELISA was not valuable in HPV positive bladder cancer identification, but the application of several probes and multiple biopsies leads

to an increased HPV detection rate in neoplastic tissues. The relationship between infection of tumour virus and high grade/high stage tumours and worse outcome revealed a negative effect of HPV infection on neoplastic tissue on behaviour and evolution of transitional cell bladder carcinoma.

An examination of 101 samples selected from urothelial bladder cancer patients did a comparison with the results generated from a morphological analysis and biomolecular detection of HPV in the samples. Of the total specimens, 38 had HPV 16 DNA, 13 had E6 mRNA and E7 oncogenes and E7 oncoprotein of HPV 16. Another notable observation was a high degree of cell anaplasia in HPV positive bladder cancer as compared to HPV negative bladder cancer. HPV was traced more often in the primary bladder tumour as compared to the recurrent bladder cancer. This study clearly demonstrated that HPV 16 is involved in the development of bladder cancer (Golovina *et al.* 2016).

Koutsky *et al.* (2002), carried out an experiment to assess the role of high oncogenic risk HPV in urinary bladder cancer development on 100 patients with bladder cancer comprising of 72 males and 28 females aged 38-90 years in Russia. A positive correlation was found between cytopathic cell changes and the antiviral antibodies level. The study concluded that HPV played a role in triggering tumour initiation in young patients with a latent infection (CMV and EBV, HSV, HPV).

A study by Abdollahzadeh *et al*. (2017), on 97 biopsy specimens at west Iran (67 experimental and 30 control) evaluated HPV infection involvement in transitional cell carcinoma. By using patients with TCC in the experiment, the study found out that HPV was positive in 22.4% of the patients suffering from TCC and 3.3% of the control group with HPV prevalence being 4.3 times higher in males as compared to females. The conclusion in this study was therefore an existence of a significant relation between HPV infection and TCC of bladder.

The etiological role in bladder carcinogenesis in fresh biopsies collected from 48 Moroccan patients (43 experimental and 5 control) demonstrated slightly more than 50% of the patients testing HPV positive. Further tests revealed HPV 16 was present in 95.5% of the experimental group. According to this experiment, the role of HPV in the genesis of bladder cancer was manifested since most bladder tumours had traces of HR-HPV genotypes, especially HPV16 (Berrada *et al.* 2013).

Heidegger *et al*. (2015), performed a study on 186 experimental tissues and 22 control tissues to elucidate HPV's role in non-muscle invasive bladder cancer by scrutinizing HPV-DNA in formalin-fixed, paraffin-embedded (FFPE) bladder cancer tissue using single-step PCR (HPV L1) for HPV detection, followed by reverse line blot (RLB) for genotyping. Low HPV infection prevalence was demonstrated only in 5 FFPE bladder cancer tissues.

A study by Shigera *et al.* (2011), on the etiological role of HPV for inverted papilloma of the bladder was examined in formalin-fixed and paraffin-embedded tissues from eight cases. Traces of HPV were detected in approximately 87.5% of inverted papilloma. In addition, 37.5% diagnosed as inverted papilloma with atypia while 62.5% were typical cases. HPV-18 and -16 were detected in 25% and 50% of the cases respectively, with 12.5% diagnosed with inverted papilloma with atypia.

All HPV positive cases were high risk and contained cellular proteins, p16-INK4a and mcm7, surrogate markers for expression of HPV-E7 with notable higher levels in inverted papilloma with atypia as compared to the typical cases.

2.10 $P16^{INK4A}$ as a Surrogate Marker for HPV Infection

The use of p16 as a surrogate marker for high-risk HPV infection is on the rise. A product of the *INK4a* gene on chromosome 9 (9p21) belonging to tumour suppressor proteins' family that is essential in cell cycle regulation, p16 accomplishes its function through suppressing cyclin-

dependent kinase-mediated phosphorylation of *pRb* to control the cell cycle at the G1–S interphase. In infected cells, the HPV E7 oncoprotein binds and deregulates host *pRb* leading to uncontrolled overexpression of p16 (Blochin *et al.* 2012).

2.11 Justification

The ability of HPV to cause malignant transformation of epithelial cells is attributed to the oncogenic activity of the virus dependent on the E6 and E7 early viral proteins whose major role is disrupting the cell cycle regulation by p53 and the retinoblastoma protein (*pRB*), respectively. HPV also leads to overall genomic instability by targeting other critical molecules such as *c*-Myc, hTERT, PDZ-containing proteins and tuberin. HPV's role for carcinomas of the cervix, vulva, penis, anus, and oropharynx has been well documented. In addition, both high-risk and low-risk types of HPV have been traced in lesions of the bladder, both benign and malignant, in several studies.

Locally, no studies have been done to show the prevalence of HPV in patients with LUT tumours. This study sought to evaluate this and to contribute to the knowledge of the possible role of HPV and lower urinary tract tumours. This was done through the use of p16 expression as a surrogate marker for HPV infection. This will further influence screening for and vaccination against HPV in the lower urinary tract.

2.12 Research Question

What is the prevalence of human papilloma virus detected in malignant urinary bladder and urethral tumours using $P16^{INK4A}$ as a surrogate marker, in the Kenyan population?

2.13 Broad Objective

To determine the prevalence of human papilloma virus in malignant tumours of urinary bladder and urethra in patients at Kenyatta National Hospital, using $P16^{INK4A}$immunostaining.

2.14 Specific Objectives

1. To evaluate and classify paraffin wax embedded urinary bladder and urethral biopsies and tissues using H&E stain.
2. To detect $P16^{INK4A}$ immunostaining (a surrogate marker for HPV) and its expression in urinary bladder and urethral tumours.
3. To determine the proportion of urinary bladder and urethral tumour subtypes, positive for HPV.

STUDY DESIGN AND METHODOLOGY

4.1 Study Design

This was a laboratory based, retrospective descriptive study.

Tissue blocks were retrieved from KNH histology laboratory archives.

Processing of the specimens was carried out at the KNH histology laboratory.

4.2 Study Population

Tissue blocks from patients whose archived histology reports were reported as being positive for malignant tumour presence. The paraffin embedded tissue blocks were then retrieved from the KNH histology laboratory from January 2014 to December 2016.

4.3 Study Eligibility Criteria

4.3.1 Inclusion Criteria

Tissue blocks reported as positive for malignant tumour of urinary bladder and urethra.

4.3.2 Exclusion Criteria

Tissue blocks excluded from this study included,

- Cases reported as positive for benign tumour of urinary bladder and urethra.
- Poorly processed tissues blocks.
- Poorly archived tissue blocks.
- Cases found to be insufficient for IHC.
- Tissue blocks that were unsuitable for processing due to disintegration.

4.4 Sample Size Determination

Calculations for the sample size was based on Cochran's (1963) formula;

$$n = \frac{Z^2 pq}{d^2}$$

Where,

n = Desired sample size

Z = standard normal distribution value (in this case Z=1.96 for 95% confidence interval)

p = anticipated true fraction (in this case 17.1% guided by Kim *et al.*, 2011)

d = precision level set at 0.05

$$n_0 = \frac{1.96^2 x\ 0.171(1-0.171)}{0.05^2} = 217$$

Currently, in Kenyatta National Hospital, approximately 30 cases of lower urinary tract tumour samples are processed and reported annually. Adjusting the sample size for finite populations less than 10,000.

$$nf = \frac{n_0}{1 + \frac{n_0 - 1}{N}} = \frac{217}{1 + \frac{217 - 1}{90}} = 64$$

Therefore, a sample size of 64 tissues was required for the study.

4.5 Sampling Method

Systematic sampling method was used. The tissue blocks were retrospectively retrieved using the histopathology files containing the reports. The laboratory number was used to identify the cases and retrieve them from the archives.

4.5.1 Sampling Frame

The sampling frame was all the cases reported as positive for malignant tumour, for the period 2014 to 2016. The records indicated a total of 91 cases of malignant tumours for this period. The calculated study sample size was 64. The samples were collected by systematic selection. A sampling interval of 2 was used to select the samples.

4.6 Materials and Methods

Demographic data (age and gender) was retrieved from the filed histology reports which were accessed from the UON Histopathology Records Office.

Rotary microtome and a microscope, provided by the Department of Human Pathology were used to section the tissue blocks.

H&E and P16 immuno-staining was done at KNH Histology laboratory.

4.6.1 Paraffin Embedded Block Retrieval

The tissue blocks were retrieved from the archives using the laboratory number on the histology reports.

4.6.2 Histological Preparation – H&E for Light Microscopy

Two to five microns sections were taken from each block, mounted on a slide labelled with a study number and then stained using standard H&E staining procedure as indicated in Appendix 1. Cases were then evaluated by the Principal Investigator and then reviewed by the supervisors.

4.6.3 Immunohistochemistry Analysis

This involved the determination of HPV positivity or negativity using the monoclonal anti-Human p16 antibody. P16 is a cellular protein that is expressed at detectable levels in HPV infected lesions.

To analyse the samples for HPV infection, sections were cut from the tissue blocks and mounted on labelled slides. These were then stained with p16 using manual immunostaining procedure as indicated in Appendix 2.

The stained slide specimens were evaluated according to a binary rating system composed of the ratings positive assigned 1 and negative assigned 0. A positive result (1) was assigned if

the p16 stained slide specimen showed a continuous/diffuse staining of cells. A negative result (0) was assigned if the p16 stained slide specimen showed no staining of the cells or staining of isolated cells/small clusters i.e. a non-continuous staining. Positive and negative controls were prepared. Positive control showed diffuse continuous staining of cells whereas negative control showed no staining or focal isolated staining.

The slides were evaluated by the Principal Investigator and then reviewed by the supervisors.

4.7 Quality Assurance

Careful selection of the paraffin wax embedded tissue blocks was done to ensure suitability of selected samples. All reagents were prepared according to the manufacturer's instructions.

Reagent expiry dates, turbidity and precipitates were checked and clearly noted. If the reagents showed any changes they were not used. Storage conditions were checked and adhered to according to manufacturer's instructions.

The kit was stored at 4-8°C according to the manufacturer's instructions. Standard operating procedures were adhered to for all procedures and strictly followed as laid down in the laboratory.

The H&E slides were well labelled and arranged in order to avoid mix up of slides and study specimens. Special slides were used for the p16 staining of samples that were mounted and fixed on these slides, in accordance to the requirements for the immunohistochemistry procedure. These were also well labelled to match the H&E stained slides to give clear correlation between the H&E and p16 stained tissue specimens.

Internal quality control was performed for all the reagents after which the staining of the samples was done.

Positive and negative controls were also prepared for the P16 procedure. Control tissues specified by the manufacturer were selected and used for this step in the procedure.

Positive control showed diffuse continuous staining of cell cytoplasm and nucleus whereas negative control showed no staining or focal isolated staining.

4.8 Data Management and Statistical Analysis

4.8.1 Data Collection

Each case was allocated a study number which was also used to label the slides. The gender and age of each case was retrieved from the histology reports and recorded in the data collection sheet (Appendix 3). Once all the tissues were re-processed and the histologic diagnosis reviewed, the data was entered into the data collection sheet. The results of P16 expression of the same, was also reviewed and this data entered. Data sheets were saved as spreadsheets in soft copies and password protected. The data sheets were filed and stored in a secure cabinet.

4.8.2 Data Analysis and Presentation

Data was entered into Microsoft Excel package, cleaned verified and password protected.

The demographic characteristics, H&E and P16 tissue descriptions were captured and analysed. Prevalence, type of tumour as well as the immunoreactivity and other categorical variables were presented as percentages. The data summary from this study was presented in tables, bar graphs and photo micrographs.

4.9 Ethical Considerations

Permission was sought from KNH/UON ERC to retrieve specimens for use in this study. All patient identifiers were protected and were not used for this study. Unique study numbers were used for confidentiality. The findings will be submitted to a scientific journal for publication.

RESULTS

Flow chart for specimen analysis

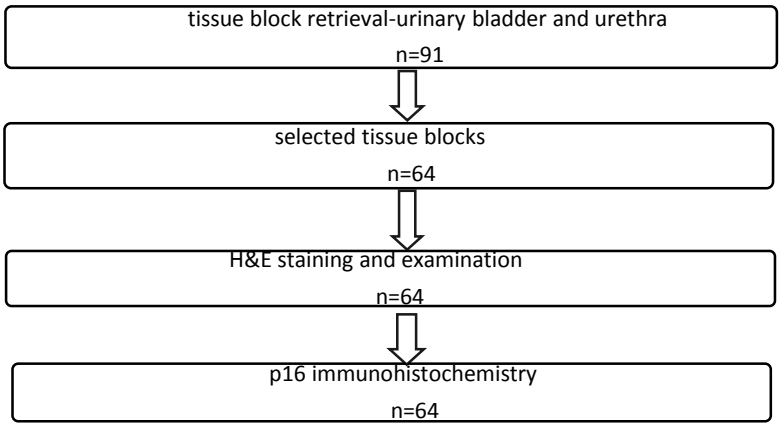

Figure 1: Specimen analysis flow chart

Demographics

A total number of 64 cases met the inclusion criteria, with 39 (61%) being male and 25 (39%) female cases. The age range was from 34 years to 80 years with a median age of 62 years. As seen in Figure 2, most of the cases (64.1%) were between the ages of 51 to 60 years and 61 to 70 years.

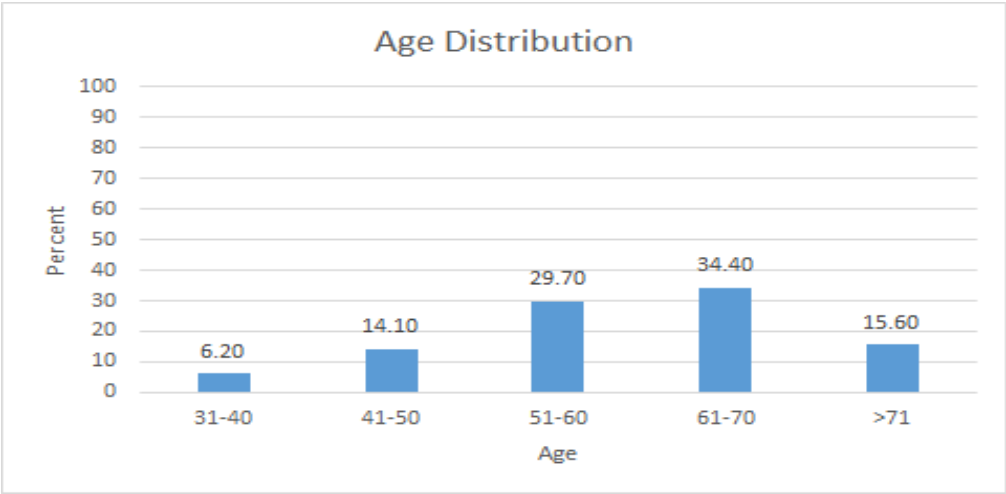

Figure 2: Age distribution

Histologic Diagnosis

A total of 64 malignant tumour cases were analysed - 61 being from the urinary bladder and 3 from the urethra. The tissues were sectioned and stained using H&E staining method and evaluated for the histological type, tumour grade and level of differentiation.

Urethral Tumours

The malignant tumours for the urethral cases included 2 invasive squamous cell carcinoma and 1 non-invasive low-grade urothelial tumour. This is shown in Table 3.

Table 3: Urethral Malignant Tumours.

URETHRAL TUMOR	GRADE/DIFFERENTIATION	NUMBER
Non-Invasive Urothelial Carcinoma	Low grade	1
Invasive Squamous Cell Carcinoma	● Moderately differentiated ● Poorly differentiated	1 1
Total		3

Urinary Bladder Tumours

Urinary bladder samples represented 95% of the cases. As per the WHO/ISUP classification, the urinary bladder malignant tumour cases were categorized as shown in Figure 3 below, with 39 (63.9%) being infiltrating and 22 (36.1%) as non-invasive.

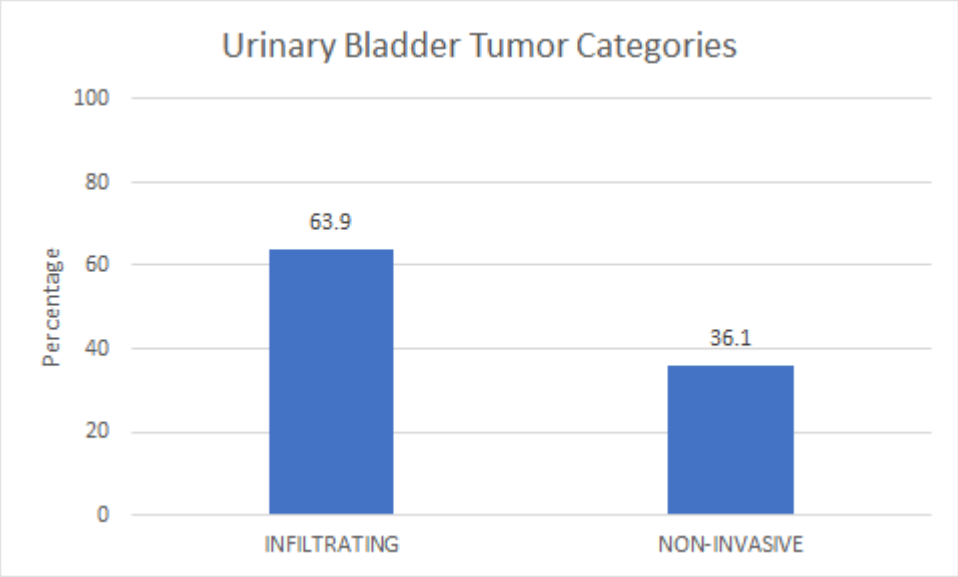

Figure 3: Graph showing classification of urinary bladder malignant tumours

Further assessment, led to the classification of both infiltrating and non-invasive tumours by histologic type. Out of 61 cases, urothelial tumours were 57 comprising 93.4% of cases. Other types were glandular 2 (3.4%), squamous 1 (1.6%) and neuroendocrine 1 (1.6%). Figure 4 below shows a graphical representation of the distribution of histological types.

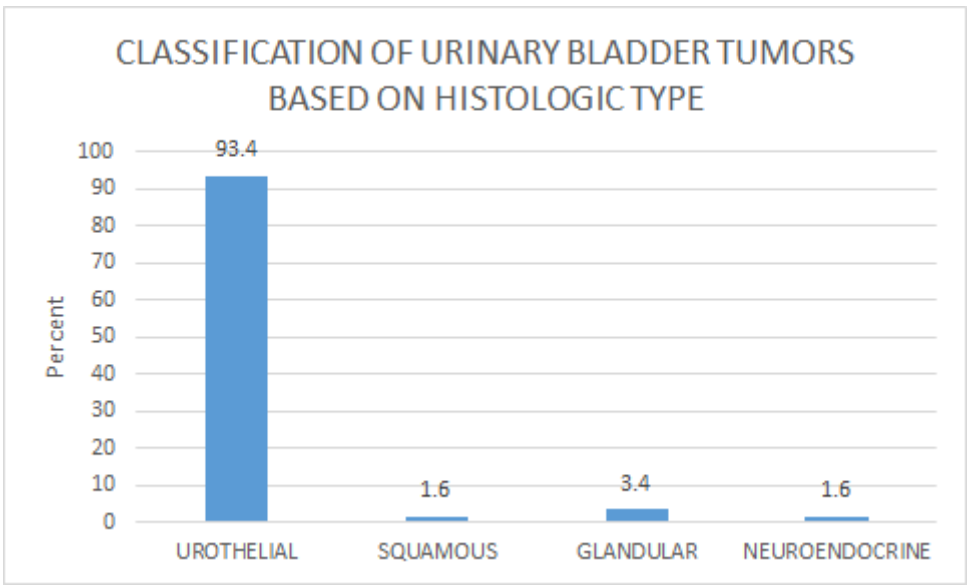

Figure 4: Graph showing urinary bladder tumours by histological type.

Urothelial Tumours

A total of 35 of the urothelial tumours were infiltrating while 22 were non-invasive. The 35 cases of infiltrating urothelial tumours were further classified according to microscopic variants. The variants included 4 (11.4%) poorly differentiated, 4 (11.4%) nested type, 2 (5.7%) sarcomatoid variant and 1 case of glandular differentiation which made up 2.9% of the cases. Twenty four, (68.6%) of the cases did not have any microscopic variation. These were classified as infiltrating urothelial tumours without any microscopic variation. The following graph illustrates these variations.

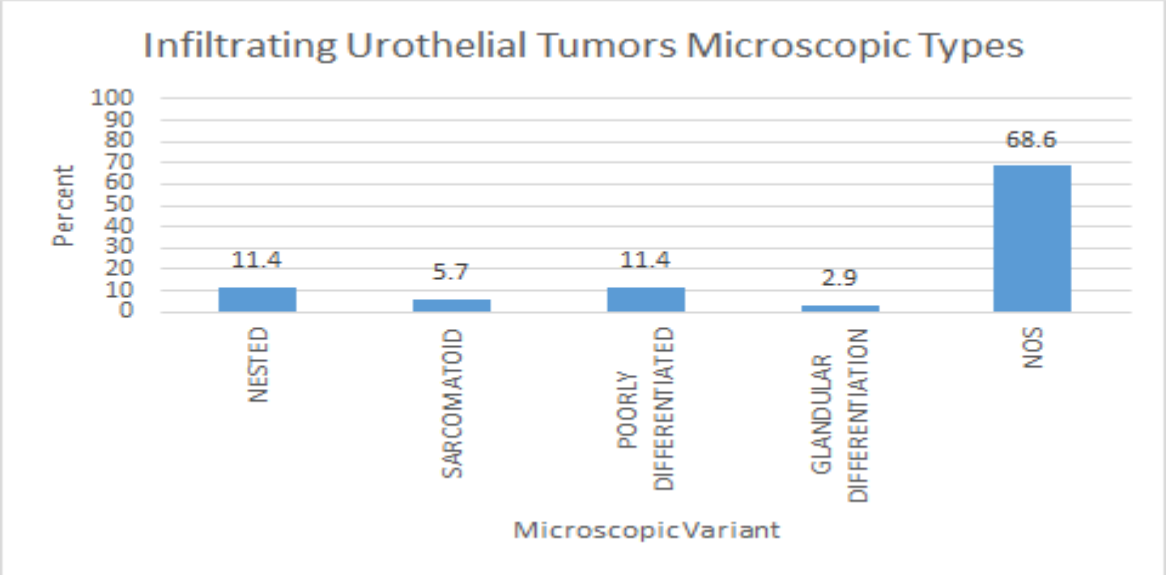

Figure 5: Microscopic variants of infiltrating urothelial tumours.

Further classification was done based on WHO/ISUP grading system, grading the 57 cases of urothelial tumours into high grade and low grade as shown in table 4 below.

Table 4**:** Urothelial carcinoma grade

GRADE	NUMBER	PERCENTAGE
LOW	24	42.1
HIGH	33	57.9
TOTAL	57	100

For the non-invasive urothelial tumour cases, 13 (59.1%) were low grade tumours while 9 (40.9%) were high grade tumours.

For the infiltrating urothelial tumours, 24 (69%) were classified as high-grade tumours while 11 (31%) were low grade tumours. The graph illustrates the grading of both infiltrating and non-invasive urothelial carcinomas.

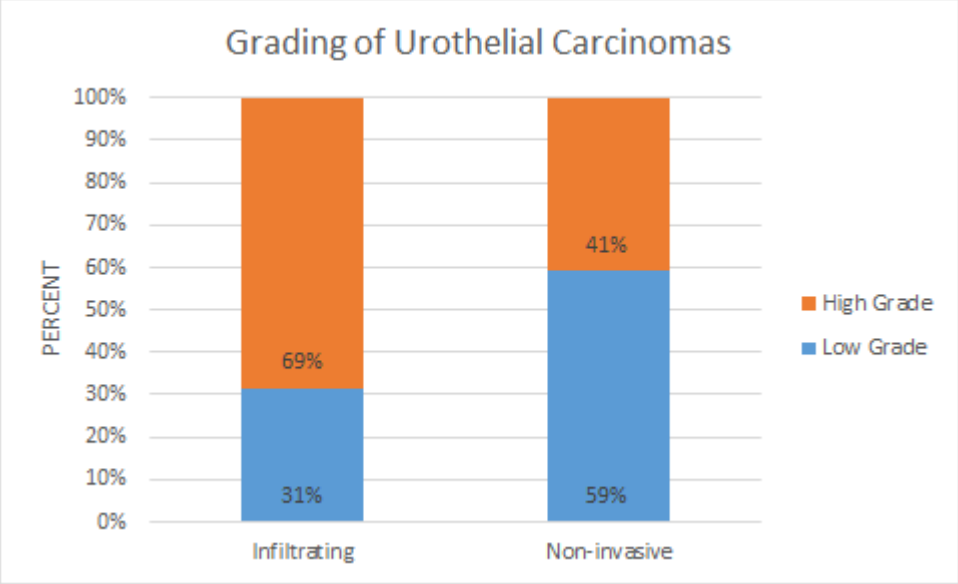

Figure 6: Grading of urothelial tumours.

Glandular, Squamous and Neuroendocrine Tumours

Of the 39 infiltrating tumours, glandular tumours made up 5.1% (n=2) cases, squamous 1 (2.6%) case and neuroendocrine was 1 (2.6%), as shown in the table 5. There were no non-invasive tumours of these histological types.

Table 5**:** Histological type of infiltrating tumours

Infiltrating Tumours	**Number**	**Percent**
Urothelial	35	89.7
Glandular	2	5.1
Squamous	1	2.6
Neuroendocrine	1	2.6
Total	39	100

Further classification of the tumours was done based on their level of differentiation. The single case of squamous cell carcinoma was poorly differentiated, the single case of neuroendocrine was small cell type while for the two cases of glandular one was well differentiated adenocarcinoma and the other case was poorly differentiated adenocarcinoma.

P16 Immunohistochemistry Staining

The 64 cases were all stained using p16 IHC stain. The results were compared to the positive and negative controls. The results showed 9 (14.1%) cases were positive while 55 (85.9%) cases were negative for p16. The cases that tested positive were all urinary bladder tumour cases as is represented in Figure 7.

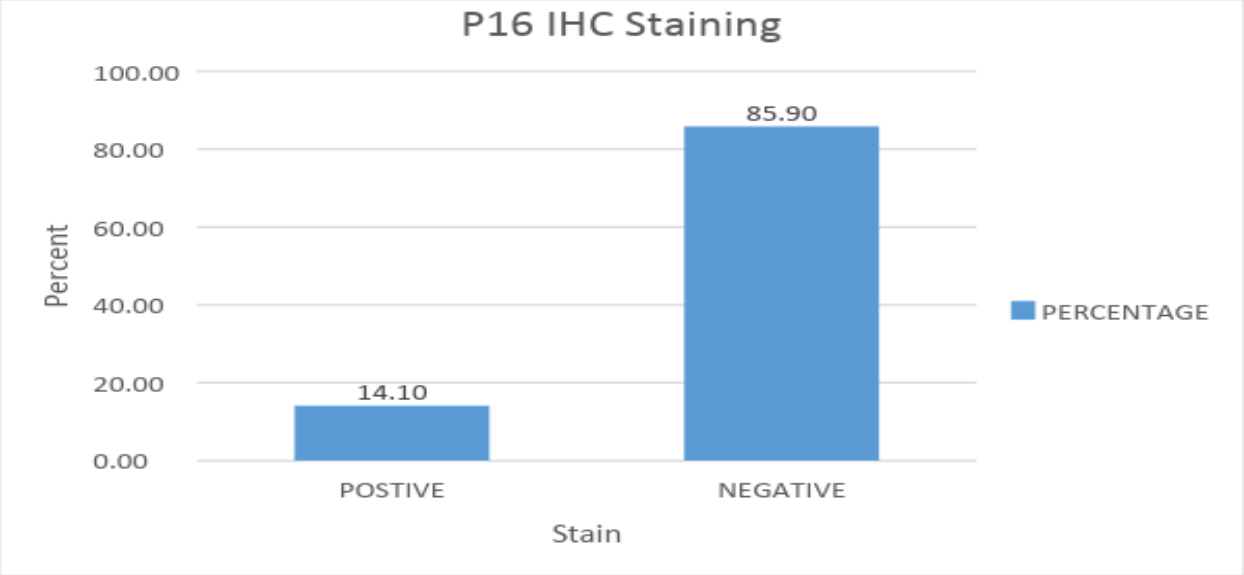

Figure 7: p16 staining of the tumour cases.

Histologic Type and Grading of p16 Positive Cases

The cases that tested positive, were categorized based on tumour type and histological type. For the cases that tested positive for p16, 4 (55.6%) were infiltrating tumours while the non-invasive were 4 (44.4%).

Table 6: p16 positive cases by tumour type.

TUMOR TYPE	NUMBER	PERCENT
Infiltrating	5	55.6
Non-invasive	4	44.4
Total	9	100.00

Based on histological type, 8 of the positive cases were classified as urothelial tumours. Of these, most were high grade tumours. The classification of the cases is summarized in the table 7.

Table 7: p16 positive urothelial carcinoma by type and grade.

UROTHELIAL P16	LOW GRADE	HIGH GRADE	TOTAL
Infiltrating	0	4	4
Non-invasive	3	1	4
Total	3	5	8

One case of squamous cell carcinoma tested positive for p16. The tumour was graded as poorly-differentiated.

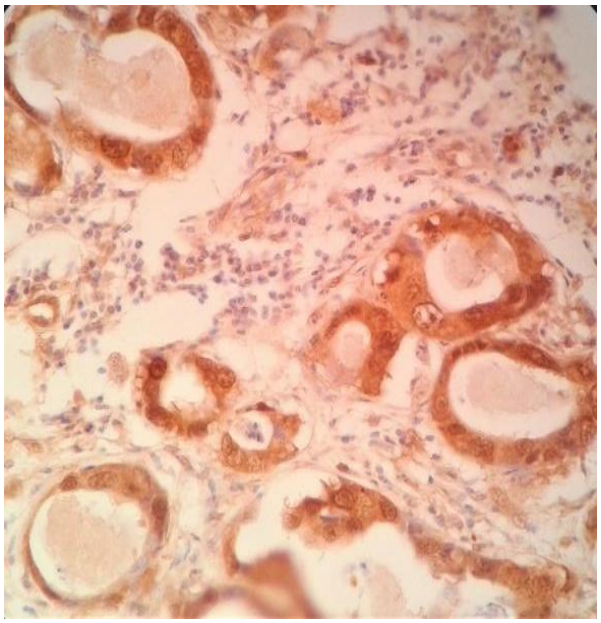

Figure 8: Positive control showing diffuse cytoplasmic and nuclear staining.

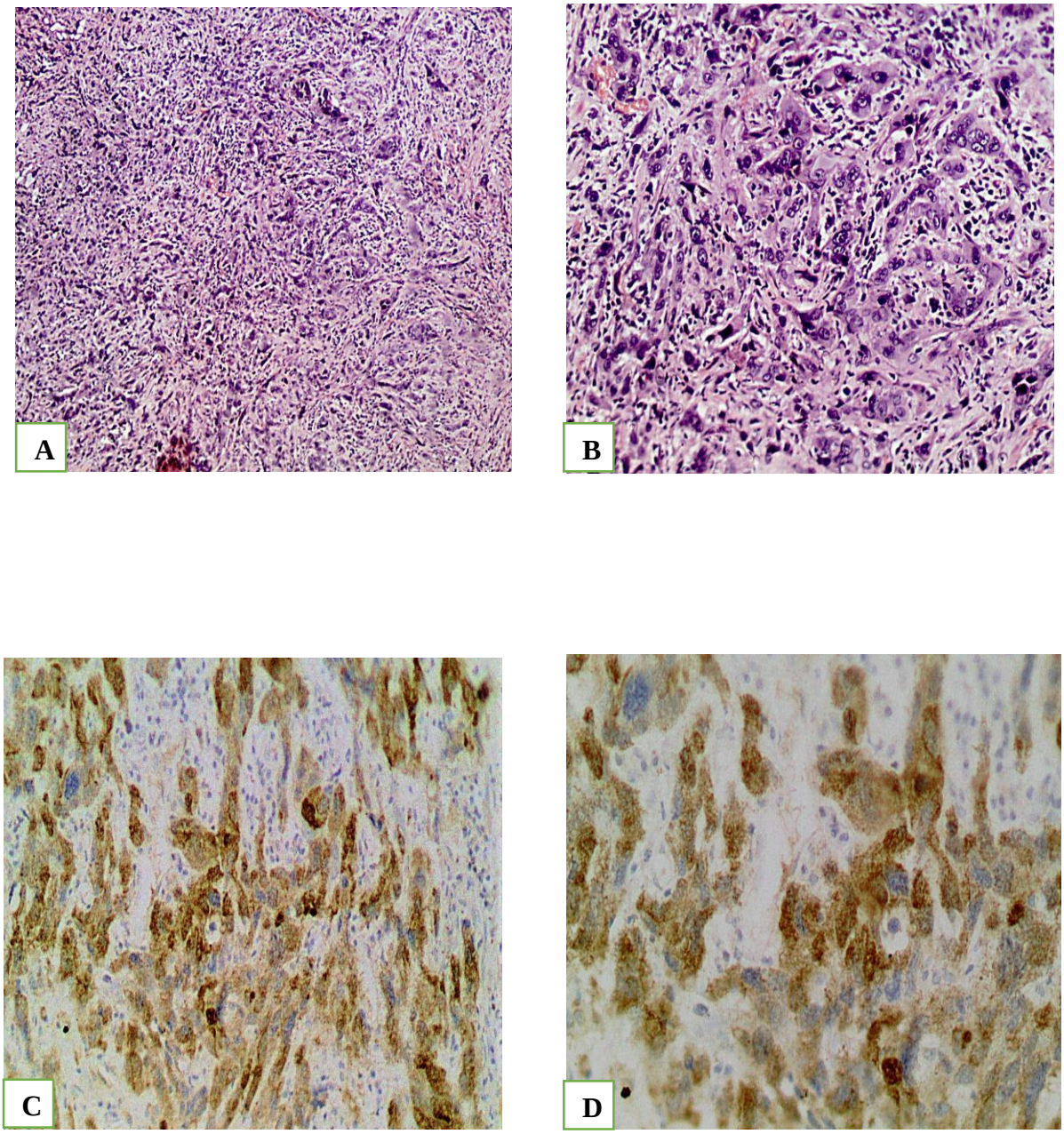

Figure 9**: CaseT19**: Invasive urothelial carcinoma, high grade-poorly differentiated variant H&E. **(A)** Infiltrating tumour within the stroma and in some areas forming small nests (x40). **(B)** Poorly differentiated tumour with pleomorphic cells, large nucleus with visible nucleoli and chromatin (x100) P16 positive: **(C)** Positive staining of the tumour cells showing diffuse pattern of staining (x40) **(D)** x100 show nuclear and cytoplasmic staining details.

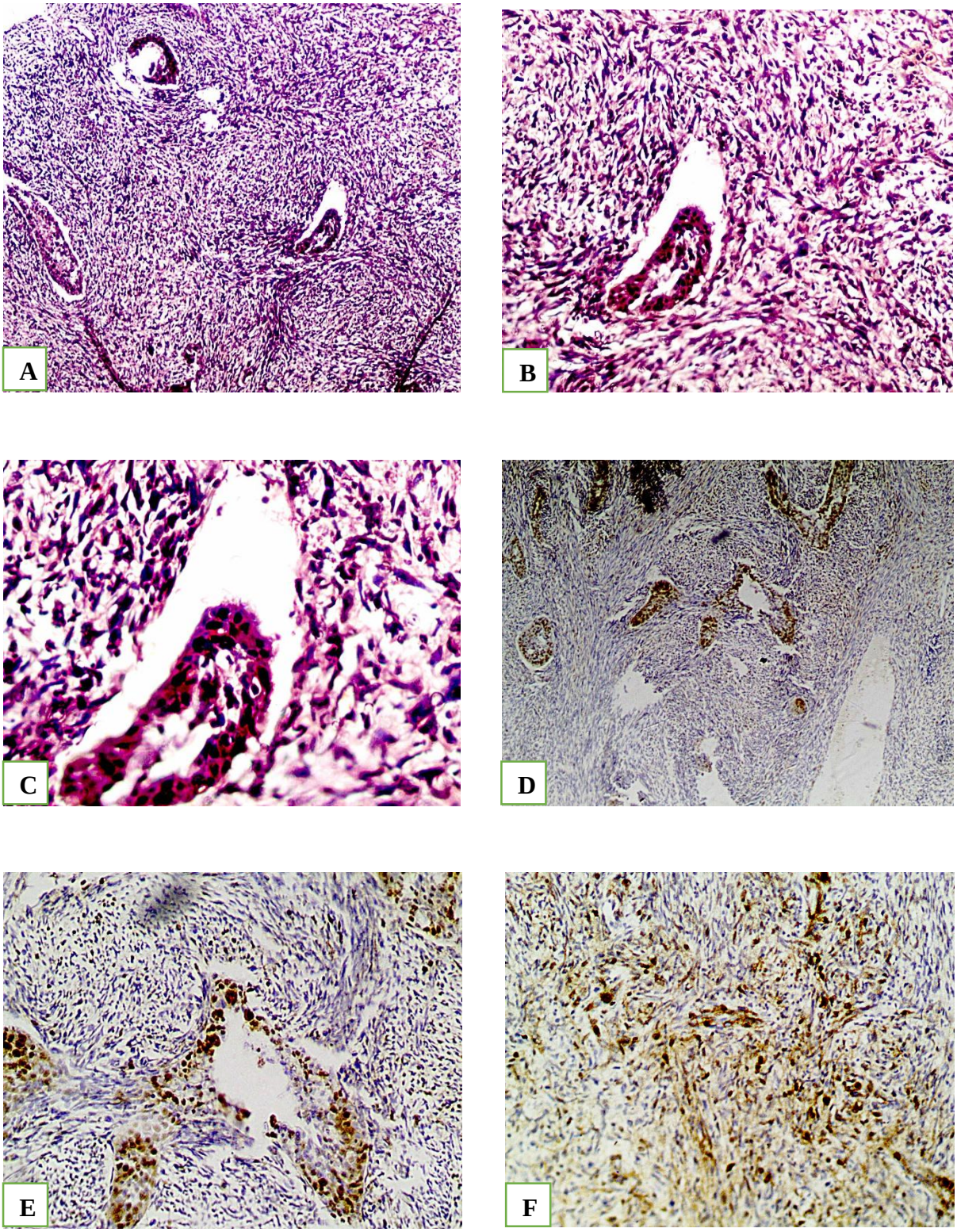

Figure 10: **Case T3**: Invasive urothelial carcinoma, high grade, sarcomatoid variant H&E. **(A)** Sarcomatoid carcinoma with both spindle and epithelial component (x40). **(B)** Invasive malignant epithelial component surrounded by malignant spindle cells (x100). **(C)** Atypical cells haphazardly arranged, nuclear pleomorphism, hyperchromatic nuclei (x200). **(D)** Positive P16 diffuse staining of tumour cells (x40). **(E)** Positive P16 staining is seen in the malignant epithelial cells (x100) **(F)** Higher magnification of the malignant spindle cells (x100).

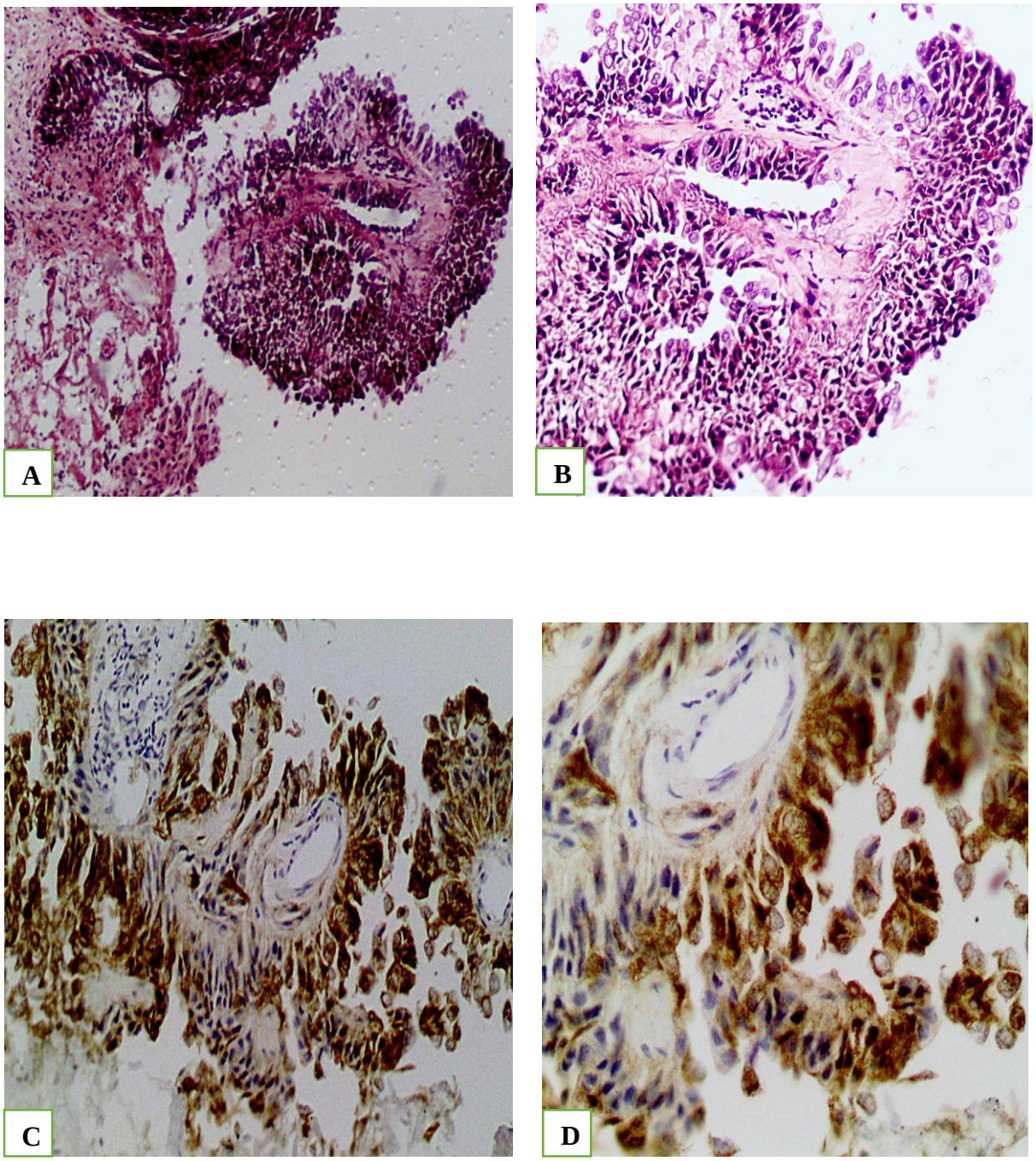

Figure 11: **Case T18**: Invasive urothelial carcinoma, high grade H&E. **(A)** Tumour with papillary pattern invading into the stroma (x40). **(B)** Tumour exhibiting haphazard arrangement of cells with variable cell layers, marked pleomorphism and eroded tumour surface (x100). P16 positive staining: **(C)** p16 staining-diffuse and continuous within the infiltrating tumour cells (x100). **(D)** (x200) showing tumour cells taking up the stain avidly.

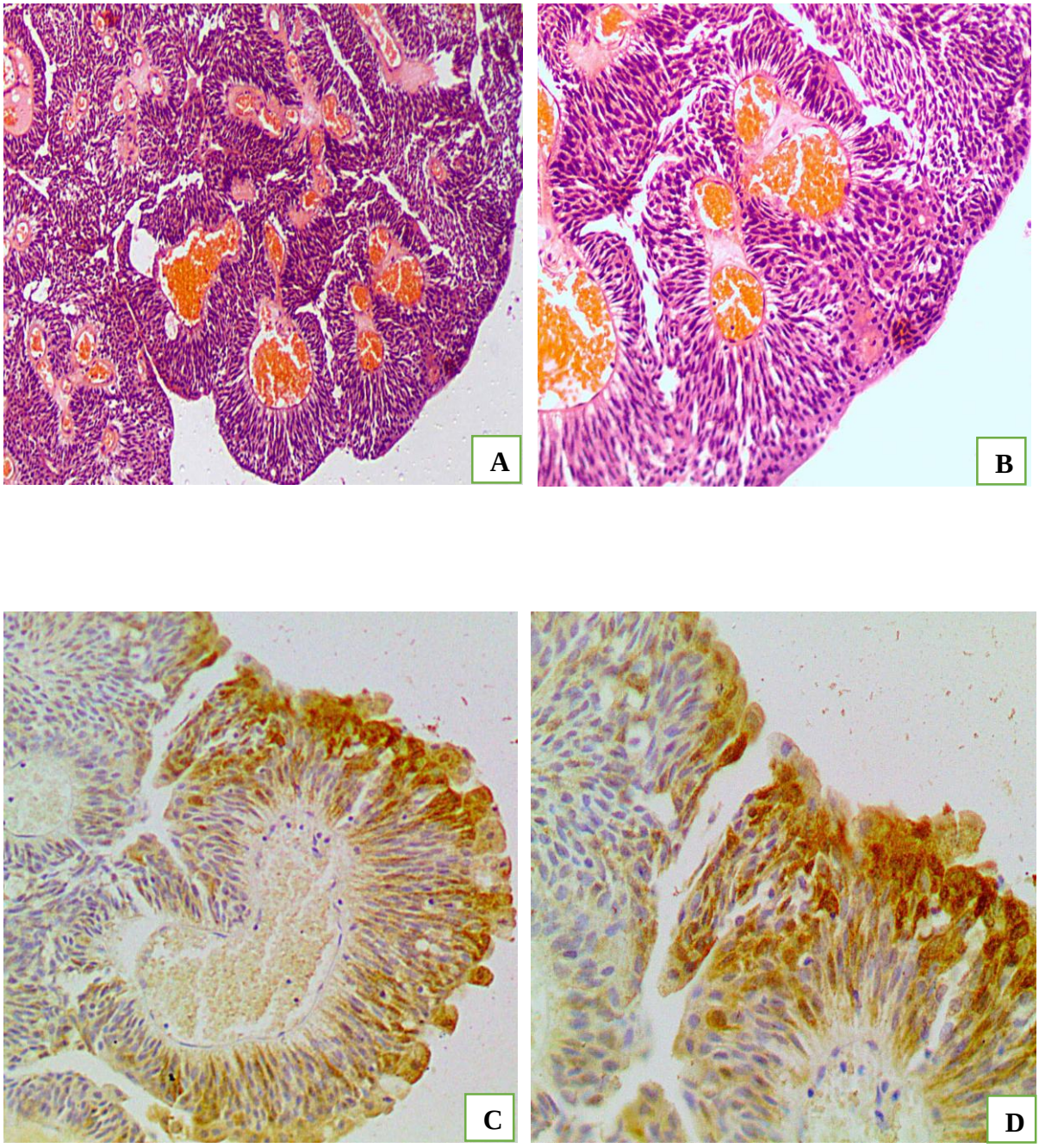

Figure 12: **Case T6**: Non-invasive urothelial carcinoma, low grade H&E. **(A)** Low grade tumour with papillary pattern surrounding vascular channels (x40). **(B)** The cells are arranged in an almost uniform pattern, minimal nuclear pleomorphism, basement membrane is intact (x100). P16 IHC staining **(C)** Positive diffuse staining of tumour cells (x100). **(D)** Positive staining of the nucleus and cytoplasm (x200).

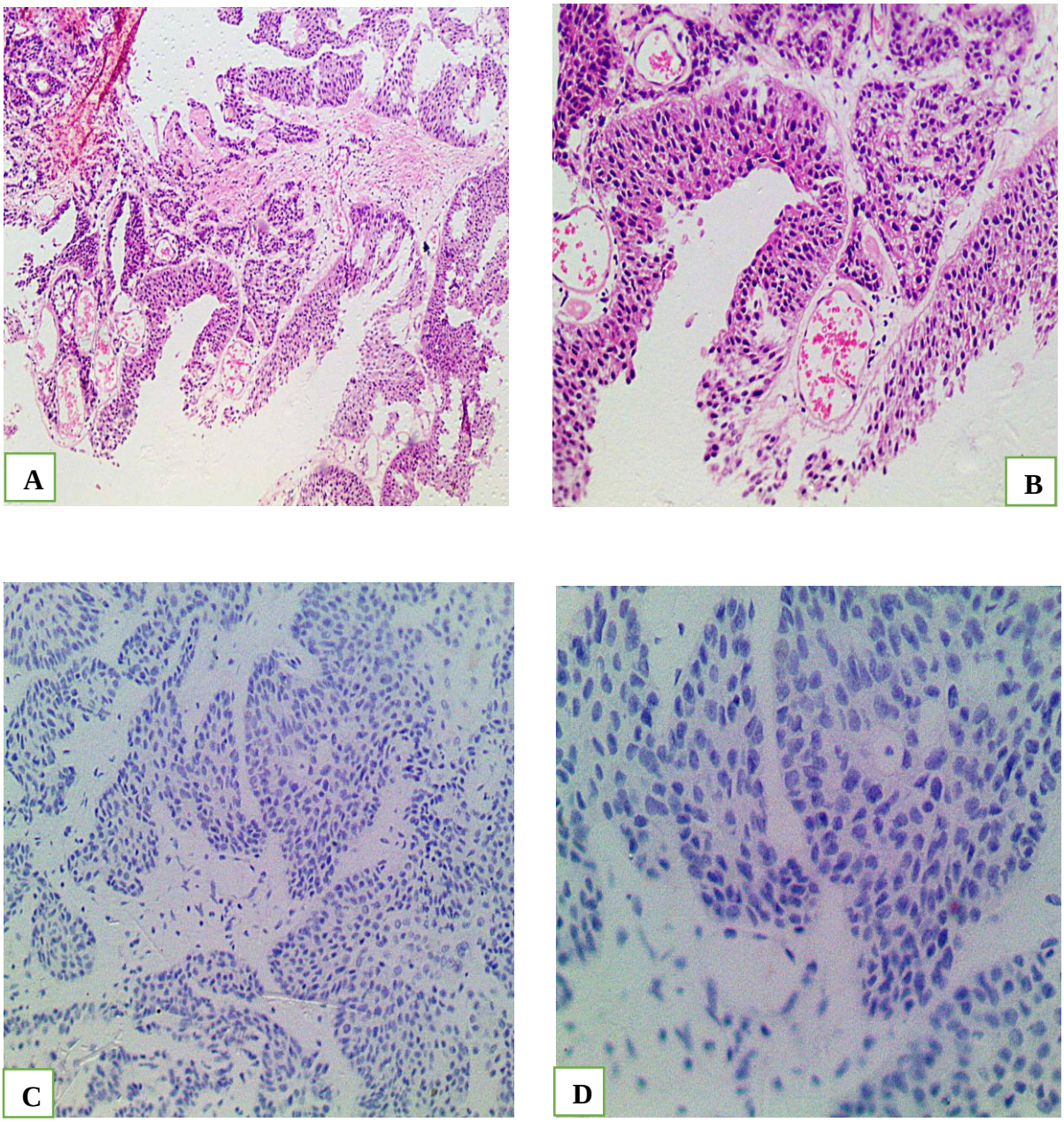

Figure 13**: Case T38**: Urothelial carcinoma, low grade H&E. **(A)** Tumour exhibits papillary architecture (x40). **(B)** Low grade tumour with cells in a predominantly orderly arrangement and mild pleomorphism (x100). **(C)** x100 **(D)** x200 P16 staining is negative.

DISCUSSION

Recently, the use of p16 as a surrogate marker for high-risk HPV infection has increased in clinical practice. According to a study conducted on p16 expression in vulvar cancers, the overexpression of p16 in HPV-transformed cells, is due to blockage of the cellular p16ink4a-CDK4-pRB downstream pathway. This blockage is due to the inactivation of *pRB* via E7 oncoprotein, resulting in the nuclear and cellular accumulation of the cyclin-dependent kinase inhibitor p16ink4a (Chapman-Fredricks, 2013).

The present study sought to establish p16 immuno-staining expression in malignant urethral and urinary bladder tumours, as a surrogate marker for HPV infection and therefore to attribute a causative role of HPV in these tumours. To do this, 64 samples from urinary bladder and urethral tumours from KNH archival material were evaluated. The samples were analysed through H&E re-evaluation and p16 IHC staining performed on them.

The cases had a male predominance of 39 cases (61%) while 25 (39%) were female cases giving a male to female ratio of 1.6:1. Several studies have shown a higher incidence of bladder and urethral cancers in males than in females. Visalli *et al.* (2018), and Chung (2013), showed similar findings. In his study on the etiology of urinary bladder cancer, Chung states that urinary bladder carcinomas ranked as the 7th most common tumour in men while in women it is the 17th most common.

For this study, the age range was from 34 years to 80 years with a median age of 62 years. Most of the cases (41 of 64) falling between 51 and 70 years. The peak age group was 61 to 70 years with 34% of the cases falling in this age group. This resonates with Chung's findings (2013) where it was determined that most cases of urinary bladder tumours occur in people older than 55 years, and 50% of cases occur in people older than 60 years.

These findings are further supported by Yaxley (2016), who found that bladders cancers mostly affect people in their 60s, and is more frequent in men than in women.

Histologic Diagnosis

The cases were stained using H&E routine staining and classified according to the anatomical site, tumour category, histologic type and grade/level of differentiation.

The most common tumour site was urinary bladder making up 95% of the cases. Bladder carcinomas are the most predominant urinary tract malignancies (Yaxley, 2016). Urethral tumours made up only 5% of the cases.

Further analysis showed that out of the 64 cases analysed, a total of 41 were infiltrating while 23 were non-invasive. The most common histological tumour type was urothelial carcinoma with a total of 58 cases (90.6%). Previous studies have shown that urothelial cancers are the most common of all bladder cancers. Other rarer cancers are squamous, neuroendocrine and adenocarcinoma. In this study, it was found that these histologic types accounted for 9.4% of the total cases, corresponding with both the Surveillance of Rare Cancers in Europe (RARECARE) project and SEER database which have reported that urothelial carcinoma of the urinary bladder and urethra is the predominant histological type (54-65%), followed by squamous cell carcinoma (16-22%) and adenocarcinoma (10-16%) (Visser, 2006). Also, a recent SEER analysis of 2,065 men with primary urinary bladder and urethral cancer found that urothelial carcinoma was most common (78%), and squamous cell carcinoma (12%) and adenocarcinoma (5%) were significantly less frequent (Rabbani, 2011).

Not surprising the majority of infiltrating urothelial carcinomas were high grade tumours at 69%, and 31% classified as low grade, while the majority of non-invasive cases were low grade 59%, and high grade tumours accounting for 41%.

p16 Immunohistochemical Staining

In this study, 14.1% of the total cases tested positive for p16. These were 9 out of 64 cases, all of which were urinary bladder cancers. None of the urethral tumours tested positive for p16. The positive p16 test is indicative of high-risk HPV infection integrated in the tumour cells as p16 is a surrogate marker for HPV infection. This results for HPV prevalence in urinary bladder cancers are almost similar to a 2007 study done by Badawi *et al.* where 15.2% of the samples tested positive for HPV infection. Of the positive urinary bladder tumours, urothelial carcinomas were eight and one case was a squamous cell carcinoma. In a study carried out by Kim *et al.* (2014), their findings agreed with other investigations in which HPV was detected in both urothelial carcinomas and squamous cell carcinomas of the bladder. Jorgensen *et al.* (2017), also reported the presence of HPV in a case of bladder squamous cell carcinoma.

On further analysis of the eight urothelial tumours, it was found that four cases were infiltrating, all of which were high grade lesions. In the 2007 study by Badawi *et al.*, they found that the infection rate of HPV types was highest in high grade carcinomas. The remaining four cases were non-invasive, 3 being low grade and 1 high grade. Shigehara *et al*. in 2011, evaluated HPV in 117 cases of bladder carcinomas. In this study, majority of the HPV-positive cancers showed a non-invasive growth pattern and most of them had been classified as low-grade. This gave a total of five high grade tumours positive for p16 and three low grade tumours positive for p16. These findings indicate that HPV infection is present in both high grade and low-grade tumours, similar to the findings of this study.

The single case of infiltrating squamous cell carcinoma positive for p16 was poorly differentiated. This finding shows p16 positivity in squamous cell carcinoma and is interpretable as a surrogate marker for HPV infection.

A study conducted by Cioffi-Lavina *et al.* (2010), supports this finding. In their study, they found 37% (14 out of 38 samples) of squamous cell carcinomas of cervix and urinary bladder tested positive for p16. They further found that p16 expression in this type of tumour is independent of gender or tumour differentiation.

The ability of HPV to cause malignant transformation of epithelial cells is attributed to the oncogenic activity of the virus. HPV's role in ano-genital and oropharyngeal carcinomas has been well documented.

From this study, the prevalence of HPV infection by use of p16 as a surrogate marker was found to be 14.1% with a higher male to female ratio (1.6:1). This can attribute high risk HPV causal role in urinary bladder and urethral malignancies in our population.

CONCLUSION

1. The use of P16 as a surrogate marker for HPV infection has aided in the identification of tumours attributed to HPV.
2. The findings demonstrate the presence of HPV in cases of infiltrating and non-invasive urothelial carcinoma using P16.
3. HPV is most likely an etiological agent of a proportion of urinary bladder cancers similar to cervical and anal cancers.

RECOMMENDATIONS

1. The routine use of molecular analysis in order to establish the actual burden of urothelial neoplasms attributable to HPV in our population.
2. The demonstration of HPV as a cause of bladder cancer is significant in the development of prevention strategies in our population. Similar to HPV vaccination for cervical cancer, a case for vaccination of boys can be included among other prevention strategies such as cessation of smoking, occupational and environmental exposure risk reduction.

LIMITATION

For tiny tissue biopsy specimens, the amount of tissue available was affected by cutting multiple sections and therefore changed the lesion seen on H&E staining and P16 IHC staining.

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APPENDICES

APPENDIX 1 - HARRIS HAEMATOXYLIN AND EOSIN STAINING PROCEDURE

Principle of the Stain

The mordant forms a lake on the tissue. It is on the lake that the stain gets attached thus colouring the cell nuclei. The nuclei having an affinity for the basic radical in the dye retains the colour even after treatment with 1% acid alcohol. Eosin stains the cytoplasm as a counter stain.

Staining technique

1. Bring section to water.
2. Stain in Harris haematoxylin for 5 minutes.
3. Rinse in tap water.
4. Differentiate in 1% acid alcohol, 3 dips.
5. Rinse in tap water.
6. Blue in Scotts tap water for 30 seconds or in running tap water for 10 minutes.
7. Counter stain in eosin for 5 minutes.
8. Rinse in tap water to remove excess eosin followed by 70% ethanol to obtain the desired shades of red and pink.
9. Dehydrate in the 3 changes of absolute alcohol.
10. Clear in 3 changes of xylene.
11. Mount with D.P.X.

APPENDIX 2 - $P16^{INK4A}$ IMMUNOHISTOCHEMISTRY PROCEDURE

1. Mount the sections on poly-L-lysine treated slides.
2. Rinse slide twice with Bond wash solution and twice with tris EDTA buffer.
3. Incubate with tris EDTA buffer for 20 minutes at 100°C.
4. Incubate further with tris EDTA buffer for another 12 minutes at room temperature. Steps 3 and 4 are also referred to as heat induced epitope retrieval (HIER). HIER describes a process of heating formalin fixed paraffin embedded tissue sections for improved immunoreactivity of tissue antigens with their specific antibodies.

Following antigen retrieval:

5. Rinse 3 times with bond wash solution.
6. Wash with bond wash solution for 3 minutes.
7. Block with peroxide for 5 minutes.
8. Rinse 3 times using bond wash solution at 35°C.
9. Incubate.
10. Rinse once with bond wash solution.
11. Apply post primary antibody for 8 minutes and wash with bond wash solution thrice each wash taking 2 minutes.
12. Apply Polymer for 8 minutes and wash with bond wash solution twice each wash taking 2 minutes.
13. Rinse with deionized water.
14. Rinse with mixed DAB refine then incubate the sections with mixed DAB refine for 10 minutes. DAB acts as the chromogen.
15. Rinse with deionized water.
16. Stain with haematoxylin for 5 minutes.
17. Rinse with deionized water.

18. Rinse with bond wash solution.

19. Rinse with deionized water.

20. Air dry.

21. Visualize $P16^{INK4A}$ expression with a light microscope.

22. Score $P16^{INK4A}$ expression.

APPENDIX 3- DATA CAPTURE SHEET

STUDY TITLE:

DETECTION OF HUMAN PAPILLOMA VIRUS USING P16^{INK4A} IN MALIGNANT TUMOURS OF URINARY BLADDER AND URETHRAL TUMORS IN PATIENTS AT KENYATTA NATIONAL HOSPITAL

STUDY NUMBER _____

STUDY SITE: KENYATTA NATIONAL HOSPITAL

DEMOGRAPHICS

1. AGE (specify in completed years)

21-30	☐	51-60	☐
31-40	☐	61-70	☐
41-50	☐	>70	☐

2. GENDER (SEX)

MALE	☐	FEMALE	☐

3. TUMOR SITE

URINARY BLADDER	☐	URETHRAL	☐

4. TUMOR

MALIGNANT	☐

5. TUMOR TYPE/CLASSIFICATION

_____

6. TUMOR GRADE

_____

7. IMMUNOHISTOCHEMISTRY - P16^{INK4A} EXPRESSION

1 POSITIVE	☐	0 NEGATIVE	☐

APPENDIX 4- KNH ETHICAL APPROVAL LETTER