VALUE OF Ki - 67 AND p63 IN GRADING OF UROTHELIAL NEOPLASMS - AN IMMUNOHISTOCHEMICAL STUDY

A Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment of the regulations for the award of the degree of

M.D PATHOLOGY – BRANCH-III

DHANALAKSHMI SRINIVASAN MEDICAL COLLEGE AND HOSPITAL, SIRUVACHUR, PERAMBALUR - 621113.

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Lastly, I am thankful to my patients without whom this study would not have been possible.

LIST OF ABBREVIATIONS

UC	:	Urothelial carcinoma
BC	:	Bladder cancer
GST	:	Glutathione s transferase
NAT	:	N –Acetyl transferase
TURBT	:	Transurethral resection of bladder tumour
BCG	:	Bacilli Calmette Guerin
MBOCA	:	4, 4’ – Methelene bis 2- chloraniline
MIBC	:	Muscle invasive bladder cancer
NMIBC	:	Non muscle invasive bladder cancer
PI3KCA	:	Phosphatidyl Inosital 3 Kinase
MAPK	:	Mitogen activated protein kinase
RB	:	Retinoblastoma gene
WHO	:	World health organization
ISUP	:	International society of urothelial pathology
PUNLMP	:	Papillary urothelial neoplasm of low malignant potential
LG	:	Low grade
HG	:	High grade
FDA	:	Food and Drug Administration
PDL	:	Programmed death ligand

IHC	:	Immunohistochemistry
SPSS	:	Statistical package for social science
HRP	:	Horse Raddish Peroxidase
DAP	:	Diaminobenzibine
X2	:	Chi – square value
P Value	:	Probability value

CONTENTS

INTRODUCTION

INTRODUCTION

Urothelial carcinoma (UC) can affect any part of the urinary tract from the collecting system of the kidney to the distal urethra. It constitutes more than 90% of the bladder cancer (BC) and is the tenth most common cancer worldwide, with an estimated 260,000 new cases occurring each year in men and 76,000 in women.[1-3] It is most commonly seen in persons over 50 years of age and occasionally in young adults and rare in children.[4] UC is approximately three to four times more common in men than females .[5-6]

Several environmental and genetic factors play an important role in pathogenesis of UC. [7-8] There is well established clear correlation of exposure to smoking and development of BC. [9] Smoking is responsible for 55% of all cases in the United States. [9-10] Following smoking, bladder cancer is most commonly seen in people exposed to industrial areas processing paint, petrochemicals and aniline dye.[10-15] Furthermore obesity increases the risk of bladder cancer by 28 %. [16]

Genetic factors like polymorphism in glutathione S-transferase gene (GSTs), which encode important enzymes involved in carcinogen detoxification. Single gene deletion of 'GSTM1 or GSTT1' or double deletions of GSTM1/GSTT1 and mutation on N-acetyltransferase 2 (NAT2) were showed to play a major role in BC occurrence. [17-19] Multiple somatic mutations are also detected in patient with UC. These mutations are identified in different grades of the tumour and highlight the potential role of genetic variations in the classification, diagnosis and treatment. [20]

Painless gross hematuria is the most common presenting complaint. The incidence of BC in patient with gross hematuria is 20%. Patients with in situ carcinoma have the symptoms of bladder irritation such as frequency and urgency. In advanced stage of disease patients may have the symptoms of obstruction and abdominal pain. [21]

Neoplastic lesions of the bladder are divided into flat and the papillary lesions. This distinction reflects two genetic pathways involved in the development of UC. Evaluation needs to assess the invasiveness (divided in to lamina propria and muscularis propria) and grade the papillary urothelial carcinoma (based on architecture and cytological atypia). [22- 23] 75% of newly diagnosed primary urothelial carcinomas are non invasive in nature and 20 % of these patients subsequently develop into invasive tumour. [24] Most of the non invasive tumours are low in grade and muscle invasive tumours are in higher grade, the former has good prognosis. [25]

UC is diagnosed mainly by transurethral resection of bladder tissues. If the biopsies do not include the muscle tissue, the invasiveness of the tumour could not be assessed histopathologically. In such cases, markers which confirm the higher grade of the tumour and potential invasiveness, may be a surrogate indicator of the risk of recurrence and progression and may help in individualized therapy.

The treatment of UC is based on histological grade and stage of the tumour. [26-28] Transurethral resection of bladder tumour (TURBT) is the primary treatment for non muscle invasive papillary carcinoma (stage $T_{a,}$ T_{is}, T_1) followed by close follow

up or immunotherapy or chemotherapy. [29- 30] Depth of tumour invasion and grade, completeness of resection and estimated risk of recurrence guide the clinician for further treatment with intravesical therapy.

Low grade T_a tumours are treated with resection alone. High grade T_a and T_1 tumours have higher risk of recurrence and progression to invasive stages and require further resection and intravesical Bacilli Calmette- Guerin (BCG). Radical cystectomy should be considered for high risk, non muscle invasive bladder cancer. [31] In situ bladder carcinoma is precursor of invasive carcinoma and treated by transurethral resection followed by intravesical BCG.

Radical cystectomy with pelvic lymphadenectomy is advised for muscle-invasive bladder cancer (stage T2 and above). [32] Neoadjuvant and adjuvant chemotherapy used in patients with muscle-invasive disease (T_3 and above), high grade histology and metastatic disease. [33]

Increased cellular proliferation correlates with biological aggressiveness of bladder tumours. [34] Ki-67 is a nuclear protein that is expressed by all cells undergoing proliferation (cell cycle G1, S, G2 and mitosis, not G0) whether it is normal or malignant and it is identified by immunohistochemically. [35- 36] A highly significant correlation of cell proliferation assessed by Ki-67 and histopathological assessment of malignancy have been reported. [37-39] Remarkable variation between Ki67 value and histological grade of the tumour has been reported, so it might be helpful to outline the individual therapy and prognosis. [40 - 42]

p63 shows varied pattern of expression in normal tissues and expressed higher level in squamous epithelium and urothelium. Expression of p63 by tumour cells indicates limited potential to progress to invasive disease and provides excellent prognosis. [43]

AIMS AND OBJECTIVES

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Primary objective:

To evaluate the expression of Ki-67 and p63 in varying grades of urothelial neoplasms.

Secondary objective:

To assess the correlation of Ki-67 labeling index and p63 expression with other morphological parameters in urothelial neoplasms.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Historical aspects:

In 1879 German urologist Maximilian Nitze invented the rigid cystoscope which completely changed the knowledge of bladder pathology. It allowed viewing inside the bladder and visualizing the growth and other abnormalities, following which, first cystectomy for BC was performed. [44- 45]

In 1940, urine cytology emerged to detect the bladder and urinary tract cancers. This test is more effective for detecting aggressive cancers than the slow growing cancers. [46]

In 1956 surgeons reported the first effective surgical technique for removing the affected bladder and surrounding tissues. But this approach carried many risks and resulted in prolonged hospitalization, hence laid the groundwork for many surgical advances in bladder cancer surgery.

An early stage of bladder cancer was noted as carcinoma in situ or non invasive flat urothelial carcinoma in 1960s & it is considered to be a precursor for invasive bladder cancer.

In 1974, a well established link between the BC and cigarette smoking was postulated. During 1980s, BCG often used for treating the aggressive form of bladder cancers to boost up the immune system and to lower the risk of recurrence. [45]

In 1978 the food and drug administration (FDA) approved the first chemotherapy drug (cisplatin) for bladder cancer. In 1984 combination of

chemotherapy drug regimen emerged which includes methotrexate, vinblastin, doxorubicin and cisplatin (MVAC Regimen). It extends the survival in advanced bladder cancer patients and remains superior to single drug chemotherapy. [47]

In 1997 combination therapy using both radiation and chemotherapy was introduced and has been an alternative treatment for patients with advanced or inoperable bladder cancers.

In 2003 pre-surgical (neoadjuvent) chemotherapy emerged, which improved the survival of patients.

In 2015, the use of immediate chemotherapy following surgery found to be beneficial for patients with advanced bladder cancers.

In 2016, FDA first approved programmed death ligand -1 (PD-L1) immune check point inhibitor - Atezolizumab (Tecentriq) for treating advanced urothelial cancers with high level of PD-L1 expression in the tumour and immune cells.

Epidemiology:

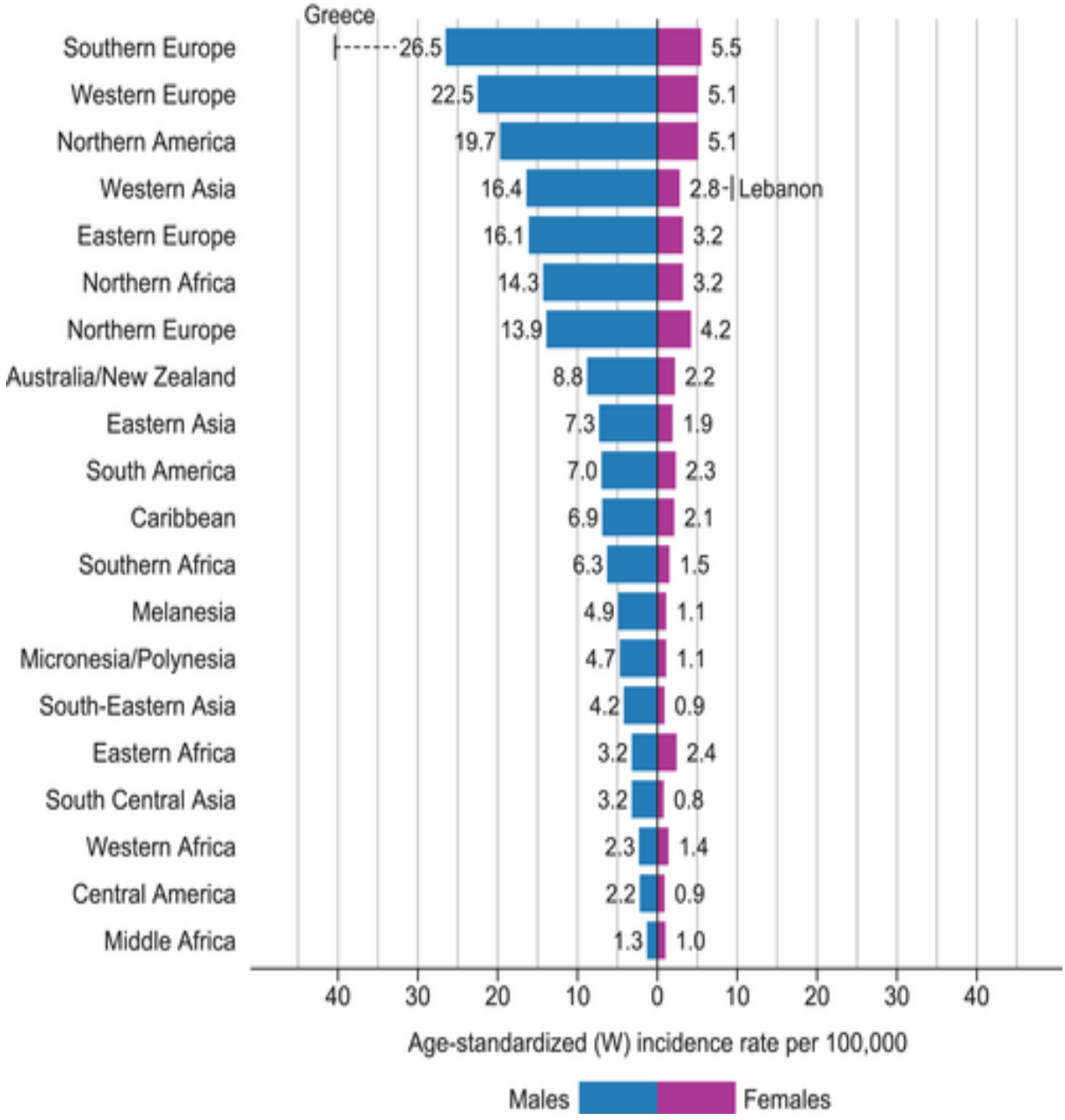
According to GLOBOCAN 2018, BC is the tenth most common cancer in the world with an estimated 549,393 new cases (3.0% of all new cancer cases) and 199,922 deaths (2.1%of all cancer related deaths). In men, it is the sixth most common cancer with an estimated new cases 424,082 (age standardized incidence rate 9.6 per 100,000 population) and ninth leading cause of cancer death with 148,270 death (mortality rate 3.2 per 100,000). In women it is seventeenth common cancer with

estimated new cases 125,311 (incidence rate 2.4 per 100,000) and 51,652 death with mortality rate of 0.9 per 100,000 population. [48]

In India according to the recent reports of the national cancer registry programme it is the ninth common cancer and is four times more common in male than females. [49] Over all incidence rate of BC is 2.5 (per100, 000 annually), 3.67 among males and 0.83 for females.

The incidence of BC increases with advancing age and is most frequently diagnosed among 65-74 years, median age at diagnosis 73 years according to surveillance of national cancer institute.

Chart: 1 Bar chart of region specific incidence age standardized rates by sex for cancer of the bladder in 2018



(Rates are shown in descending order of the world ASR among men and the highest national rates among men and women are superimposed (GLOBOCAN 2018)

Geographical and racial distribution of BC varies in different parts of the word and more commonly seen in American countries than in Asian population. This indicates various etiological factors influence the development of BC. [50] The incidence of smoking among males explains the male predominance of BC. [51- 52]

Age, gender and racial factors will affect the prognosis and survival of the patient. [53] More than half of the newly diagnosed patients with BC are in high grade with muscle invasive cancer at the time of diagnosis. [54] Most of the younger patients have low grade and stage of tumour and behave in indolent fashion. [55] Cancer stage at the time of diagnosis will determine the treatment options and has a strong influence on the length of survival. If the BC diagnosed in early stage, the 5 year survival rate is 77.1%. [56]

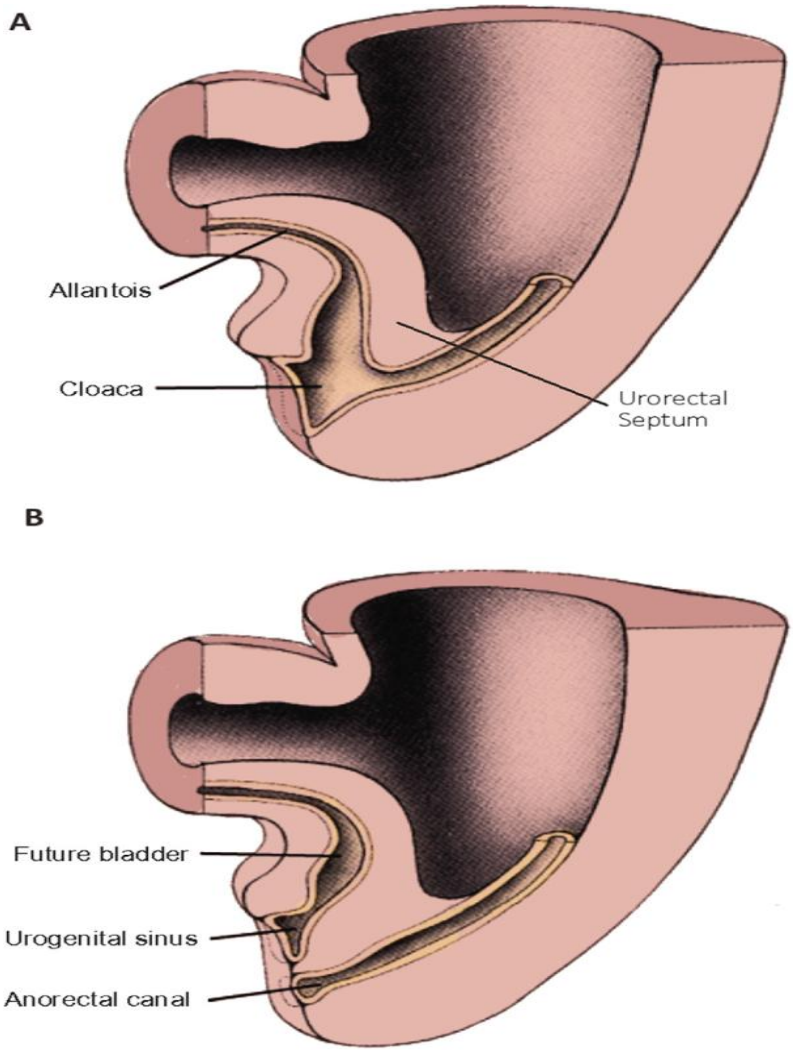
Embryology:

Development of urinary bladder starts at twelfth week of intrauterine life. Cloaca is partitioned into the primitive urogenital sinus and the anorectal canal. The primitive urogenital sinus is in continuity with the allantois. Bladder forms from primitive urogenital sinus above the confluence with wolffian duct (mesonephric duct) which serve as the demarcation between cranial vesico urethral cannel and caudal urogenital sinus. Most of bladder parts derived from the cranial part of the urogenital sinus and trigone developed from caudal end. The epithelium of bladder is derived from endoderm of the urogenital sinus, while lamina propria, muscularis propria and adventitia develop from the surrounding splanchnic mesoderm. [57] (Figure.1, Figure.2)

Anatomy:

The bladder is a muscular organ, hallow in shape situated at the base of the pelvis. It is wider at superior aspect and narrow towards the inferior region and creating the inverted pyramid in shape. Anatomically bladder is divided in to dome (superior surface), base (posterior surface) and two inferolateral surfaces. Triangular region at the base of the bladder is called trigone. It is bounded posterolaterally by the ureteric orifices and inferiorly by internal urethral orifice. [58] (Figure.3, Figure.4)

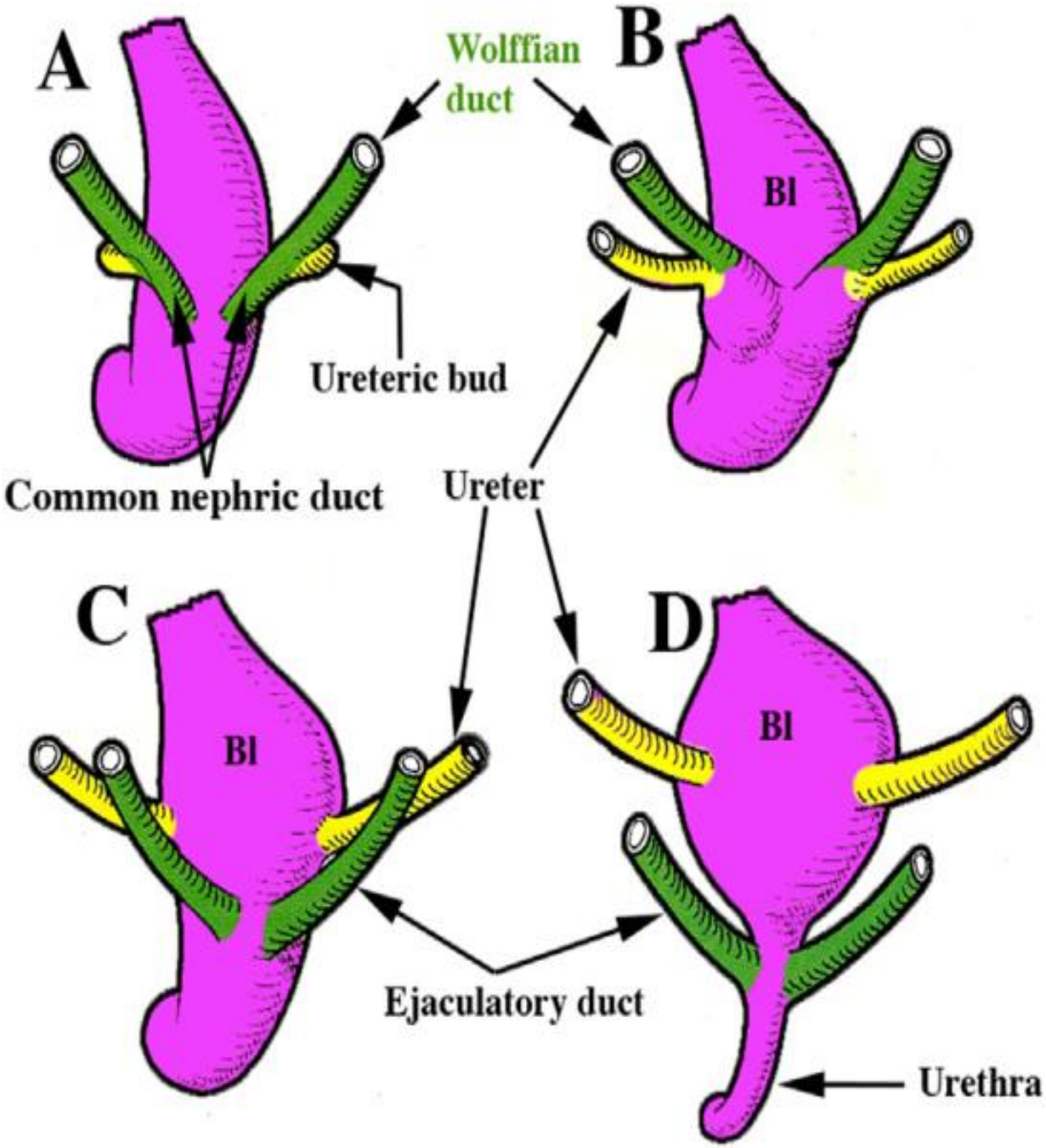
Figure: 1 Development of urinary bladder



A) Division of the cloaca into urogenital sinus and anorectal canal by urorectal septum.

B) After division of the cloaca into urogenital sinus and anorectal canal.

Figure: 2 Development and remodelling of ureter and wolfian duct



A) Origin of the ureteric bud from the Wolffian duct
B) Remodelling of the positions of the ureters and Wolffian ducts (ejaculatory ducts) in male embryos (B-D).

Figure: 3 Anatomy of female urinary bladder

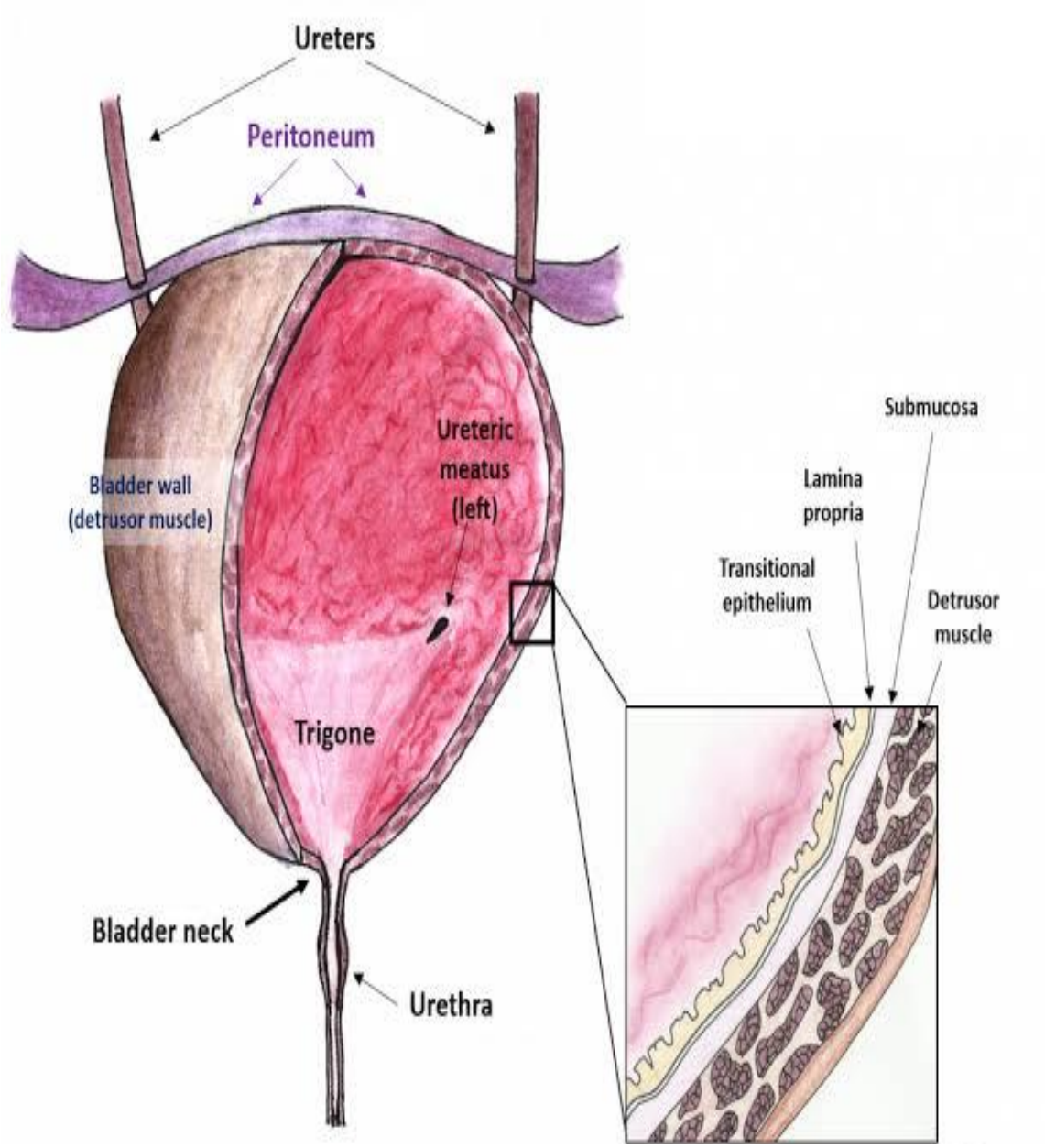
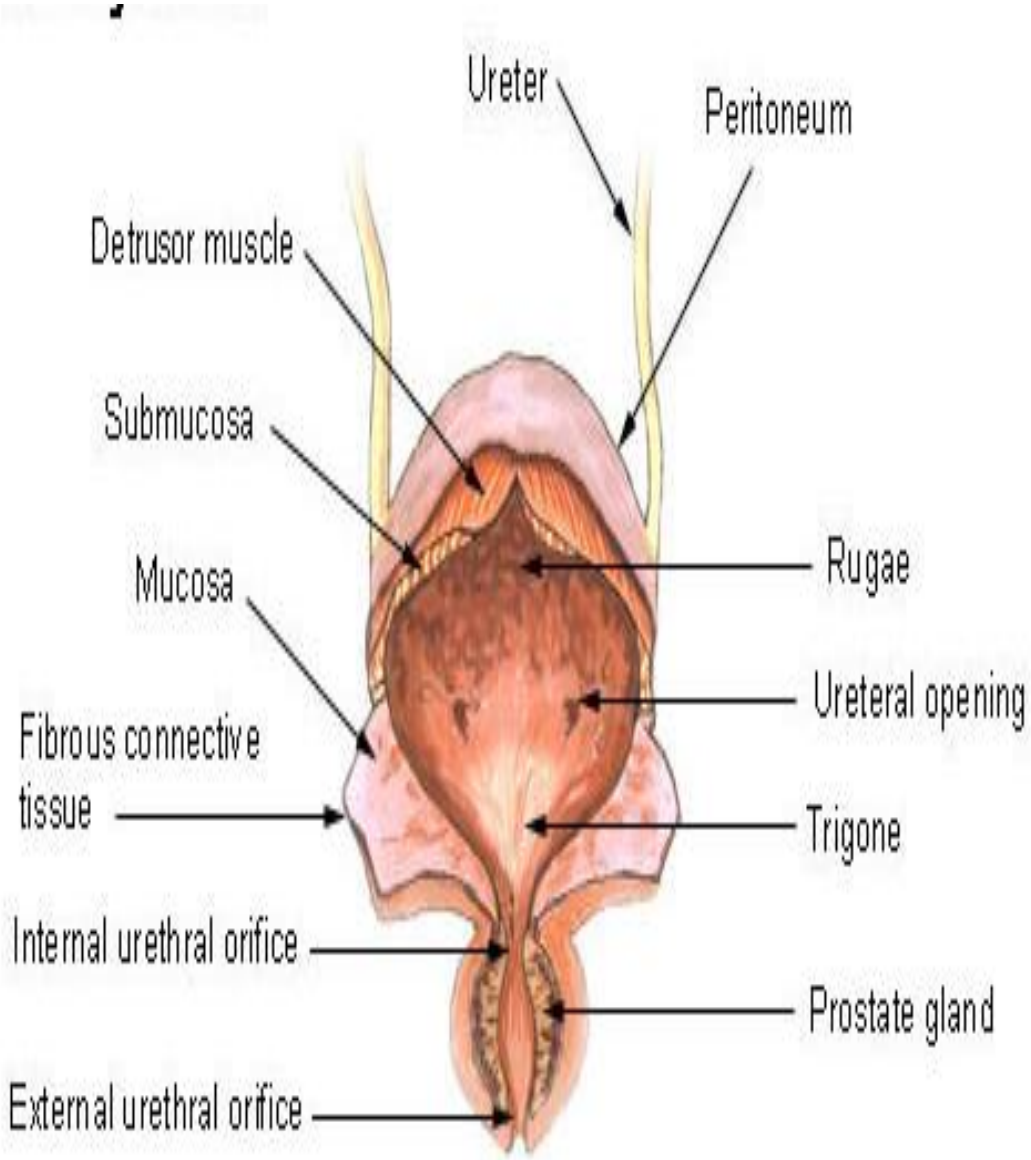


Figure: 4 Anatomy of male bladder



Histology:

The wall of the bladder has three layers, from inside to outside they are i) Mucosa (urothelium, lamina propria, muscularis mucosa) ii) Muscularis propria iii) Adventitia or serosa. [59] (Figure. 5)

Urothelium:

The thickness of the urothelium varies, which is 5- 7 cell thick in contracted bladder, 2-3 cell thick while in distended bladder. Superficial layer of urothelium consist of single layer of umbrella cells. The cells are large and having abundant eosinophilic cytoplasm with prominent nucleoli. Some of the cells have binucleation also. Intermediate layer of urothelium composed of several layers cuboid to low columnar cells. The cells are having well defined cell borders with amphophilic cytoplasm; regularly arranged oval nuclei, long axis perpendicular to the basement membrane; finely granular chromatin, no mitosis. Basal urothelial cells are small cylindrical and lies on the basement membrane, sometimes have longitudinal grooves. (Figure.6)

Lamina propria:

Lamina propria lies between the mucosal basement membrane and the muscularis propria. It is composed of abundant loose connective tissue rich in blood vessels, lymphatic channels, sensory nerve endings and adipose tissue. It is thinner in trigone of bladder and neck.

Muscularis propria:

Muscularis propria consisting of three layers of thick muscle bundles, inner longitudinal, middle circular and outer longitudinal bundle. It also contains adipose tissue and paraganglia in between the muscle fascicles.

Figure: 5 Histology of urinary bladder

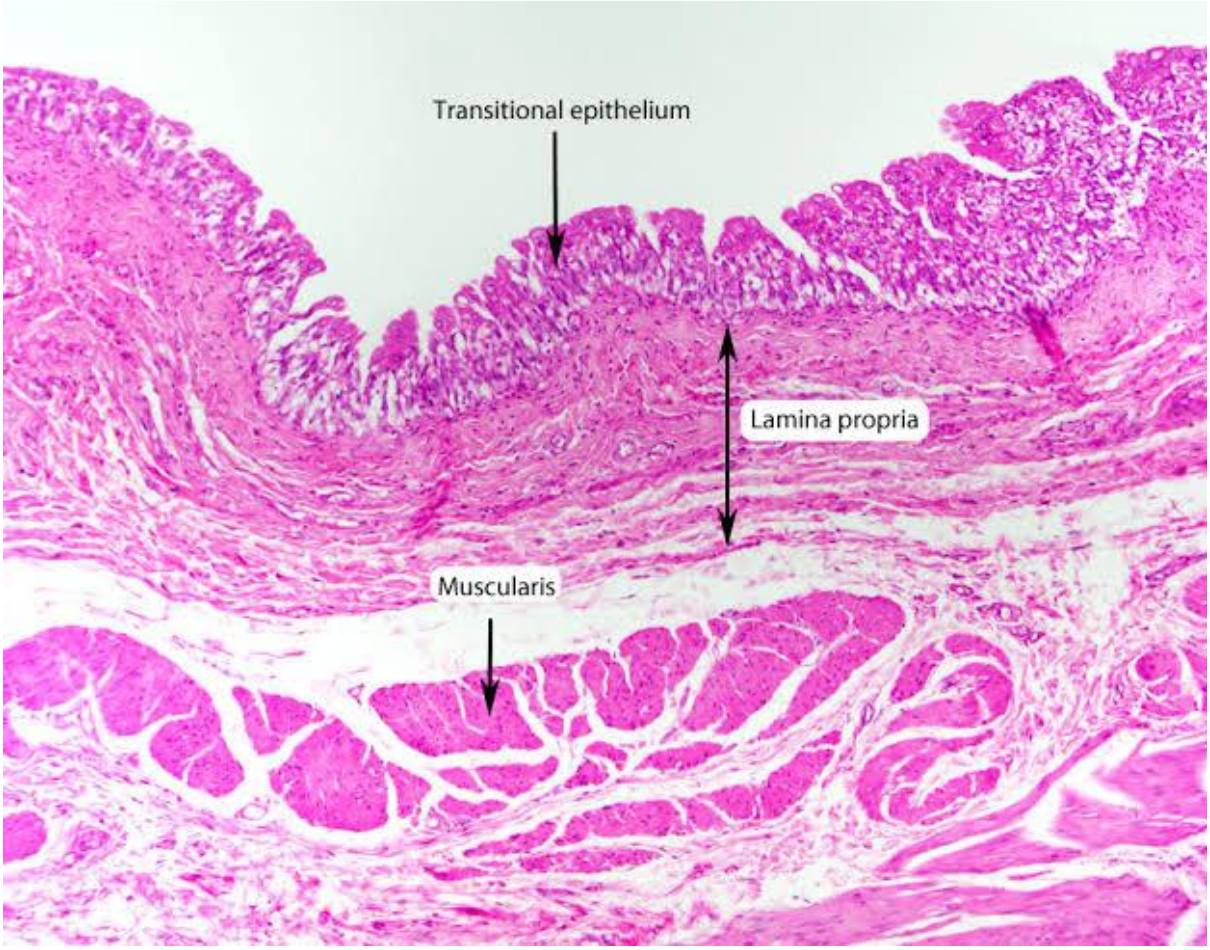
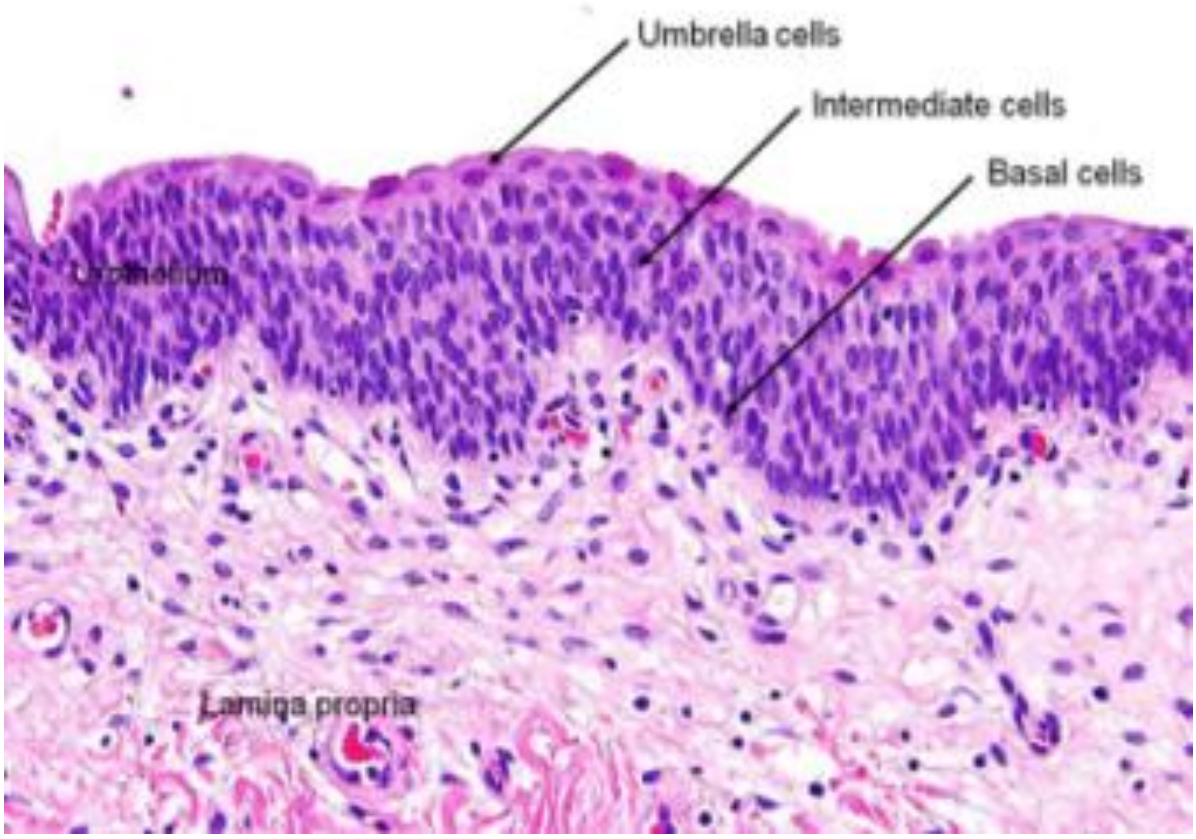


Figure: 6 Layers of urothelium



Risk factors for bladder cancer:

Multiple etiological factors play a role in development of bladder carcinoma. These include cigarette smoking, occupational exposure of certain chemicals and infections.

Tobacco smoking accounts for 50% of the cause. [60] Smokers have a four times higher risk for getting BC compared to non smokers. The number of cigarettes and duration of smoking is associated with increased risk in both sexes. [61- 62] The exact mechanism is not known but certain chemicals found in the cigarettes such as naphthylamine- 2, aminobiphenyl-4 and polycyclic aromatic hydrocarbons increase

the risk. [63] They have direct mutagenic effect on epithelium and this genetic damage causes uncontrolled cell growth and inhibition of tumour growth inhibitory mechanisms thus leads to cancer development. [64- 65]

Exposure of occupational carcinogen accounts for 10% of BC and occur mainly through skin contact. Aromatic amines such as benzidine and beta naphthylamine are used in dye, rubber and plastics manufacturing industries, paint products and fungicides which are carcinogenic. It will cause multifocal with higher stage of BC. Cigarette smoking along with occupational exposure of work place chemicals was act together to cause BC. [65] 4, 4′-Methylene-bis (2-chloroaniline) (MBOCA) are a therapeutic agent used in polyurethane products. Absorption of vapour or dusts of MBOC increase the risk of BC. [66]

High level of arsenic in drinking water has been linked with a higher risk of bladder cancer in some parts of the world like India, china and Hungary. [67]

Bladder stones, urinary tract infection and prolonged bladder catheterisation cause chronic irritation which leads to development of bladder cancer. Schistosoma haematobium infection associated with increased risk of development of squamous cell carcinoma rather than transitional cell carcinoma.

Exposure of ionizing radiation and cyclophosphamides increase the risk of bladder carcinoma with dose and duration dependent pattern.

Certain nutritional factors like lake of fluid intake [68], vitamin D deficiency [69] and food rich in aristolochic acid also increase the risk. [70]

Genetic predisposition also influences the incidence of BC. First degree relatives have higher risk. Hereditary genetic changes such as mutation in acetylator gene N-acetyltransferase 2 (NAT2) and glutathione S-transferase mu1 (GSTM1) leads to difficult to decompose some of the toxins in the body and thus lead to BC development.

Other risk factors:

- Whites are likely to develop the bladder cancer twice higher than blacks
- The risk of bladder cancer increases with age. About 9 out of 10 people are older than 55 years.
- Bladder cancers more common in men than in women.

Clinical presentation:

Painless macroscopic haematuria throughout the micturition is the most common presenting symptom. If the patients have macroscopic haematuria the incidence of BC was 20%, while in the presence of microscopic haematuria the incidence only 2%. Bladder carcinoma in situ lesion causes urinary urgency and frequency. If the tumour located close to the urethra causes symptoms of obstruction. Advanced tumour leads to abdominal pain, bone pain and ureteric obstruction due to metastasis.

Molecular pathogenesis of urothelial carcinoma:

UC have diverse morphologic and clinical manifestations. [71] Superficial and muscle-invasive urothelial carcinomas are two distinct types of the tumour have different biological behaviour and prognosis. Molecular evidence also supports the different pathways of pathogenesis in those tumours.

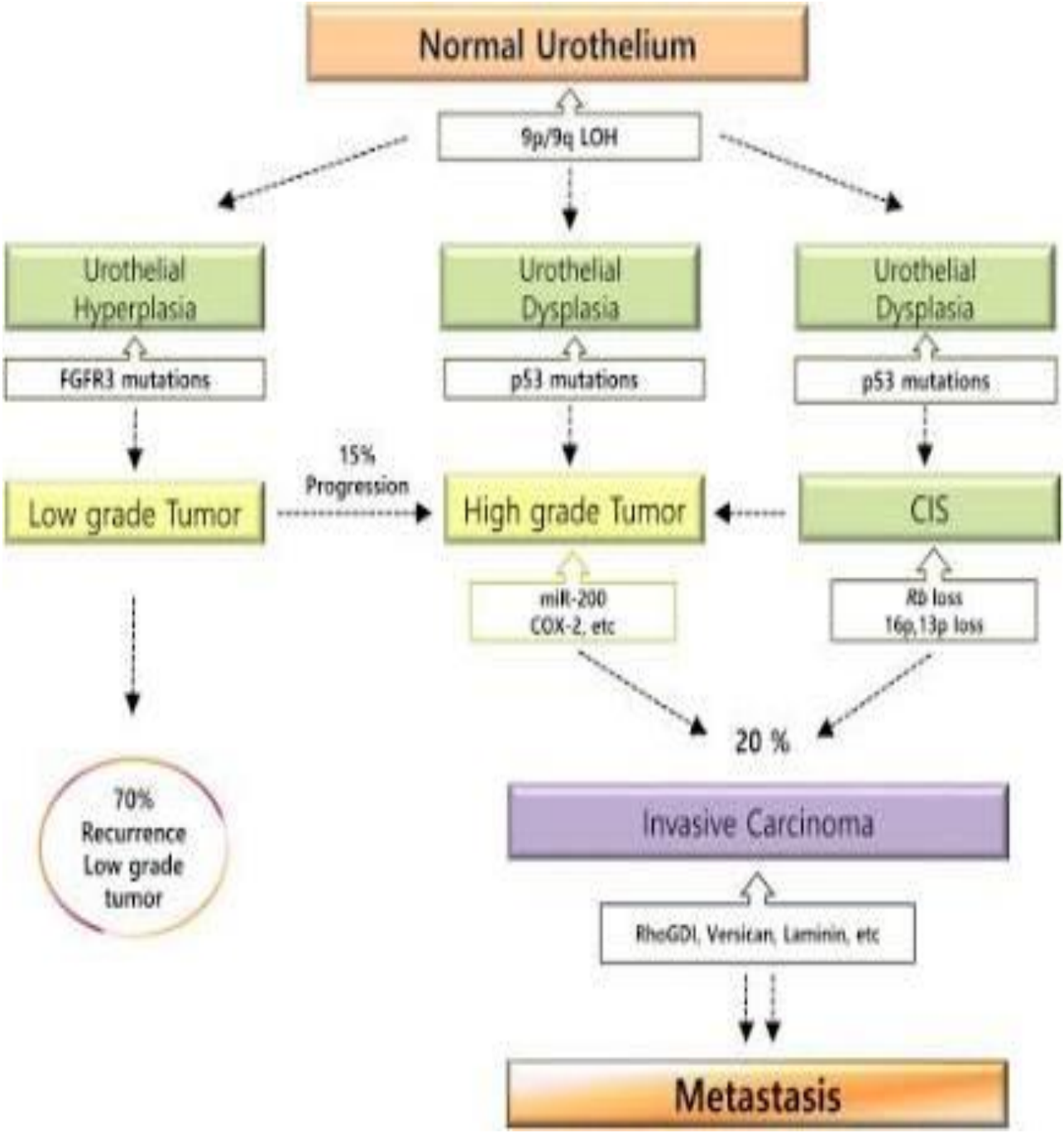
More than 90% of superficial urothelial carcinoma arises from urothelial hyperplasia, out of which 15% progress to high-grade non invasive and subsequently invasive UC. Invasive carcinoma arising from progression of dysplasia to flat carcinoma in situ (CIS) and high-grade non invasive UC, further accumulation of genetic alterations leads to progression to muscle-invasive bladder cancer (MI-BC).[72-73] Alteration in chromosome 9 is the earliest genetic defect responsible for providing the milieu for accumulation of genetic instability.

Transformation of normal urothelium in to superficial non muscle- invasive bladder cancer (NMI-BC) involves the genetic alterations includes fibroblast growth factor receptor -3(FGFR-3), H-RAS, and Phosphatidyl Inositol 3 kinase oncogene (PI3KCA) [74-77]. Activating mutation involving H-RAS gene on codon 12 and FGFR-3 are found 15%, 80% of the NMI-BC respectively. Activating mutations in RAS lead to the activation of mitogen activated protein kinase (MAPK) and PI3K pathways. Mutation in Inhibitor of cyclin dependent kinase 4 (INK4) genes and deletion of 9q associated with higher grade of tumour and important in progression of lower grade to higher grade.

The pathogenic pathways involved in MI-BC are alteration in tumour suppressor genes including p53, p16, and Retinoblastoma gene (RB).Deletion of RB gene commonly seen in high grade tumour and associated with aggressive clinical course. Missense mutations of TP53 lead to an altered protein which is resistant to degradation through the ubiquitin pathway resulting in nuclear accumulation. This leads to deregulation of progression of the cell cycle through G1-S checkpoint and allow for cancer development and progression through altered apoptosis, DNA repair and response to therapy. [78]70% of UC have TP53 mutation associated with higher grade, stage and poor clinical outcome.

Somatic chromosomal alterations such as gain of 3q, 7p, and 17q and deletion of 9p21 have diagnostic and prognostic value. The increasing tumour grade and stage have been associated with copy number abnormalities, loss of heterozygosity and increased genomic instability.

Chart: 2 Pathogenesis of urothelial carcinoma



WHO/ISUP classification urothelial carcinoma (2016):

Several classification systems for UC have been introduced over the years; most widely accepted system was world health organization (WHO) 1973 system. [79] Due to poorly defined morphological criteria, no distinct cut off between different

grades of the tumour this system lake the reproducibility. This system includes the grading such as G1/2 and G2/3 , it does not give clear guidance to the clinicians regarding the course of treatment.

The WHO /International society of urological pathology (ISUP) 1998 creates a universally acceptable classification system for bladder neoplasia that could be used effectively by pathologist, urologist and oncologists. It distinguishes the papillary neoplasm in to papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), and low grade (LG) and high grade (HG) urothelial carcinomas based on morphology. It also introduces the definition for hyperplasia and flat lesions.

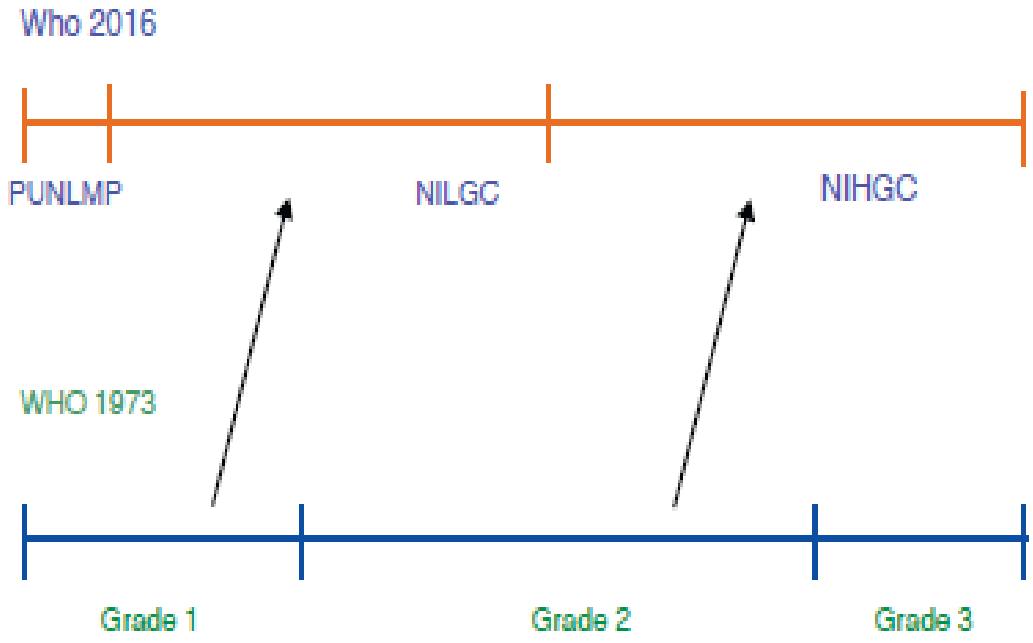
The WHO 2016 system is based on the WHO/ISUP 1998 classification and the WHO 2004 classification. 2016 classification provide strong points of clear cut-offs between HG and LG tumours, along with clear, precise descriptions of each grade in order to obtain homogeneous groups of tumours. It avoids the use of ambiguous grading, G1/2 or G2/3. According to the WHO 2016 system, pTa and pT1 tumours are graded into LG and HG and all detrusor muscle invasive urothelial carcinomas are considered to be HG tumours. The decision to stratify pT1 carcinoma as LG was taken because while they often recur they show progression in only 5% of cases. [80] LG and HG pTa carcinoma shows significant difference in recurrence rate but does not correlate with disease progression. They pointed out the problem of both tumour categories being treated with a range of different treatments, as well as the frequent overtreatment of patients.

The term of non invasive carcinoma has also been introduced in the WHO 2016 system and to further differentiate non invasive LG and HG papillary carcinoma

from invasive urothelial carcinomas. In 2016, the newly described or better defined non invasive urothelial lesions include urothelial dysplasia and urothelial proliferation of uncertain malignant potential, which is frequently identified in patients with a prior history of urothelial carcinomas. Category of papillary neoplasm (PUNLMP) has a negligible risk of progression although the potential for recurrence requires some level of clinical follow-up.

It also defining a group of lesions with a high risk of progression and those candidates are chosen for adjuvant and neoadjuvent therapy recommended by ISUP, WHO, ICCR.

Chart: 3 Comparison of the WHO 1973 and 2004/2016 classification of urothelial carcinoma



NILGC – Non invasive low grade papillary urothelial carcinoma
NIHGC – Non invasive high grade papillary urothelial carcinoma
PUNLMP – Papillary urothelial neoplasms of low malignant potential

Classification of bladder cancer:

Histological classification of tumours of Urothelial tract

(WHO 2016)

Urothelial tumours

- Infiltrating urothelial carcinoma:
    - Nested ,including large nested
    - Microcystic
    - Micropapillary
    - Lymphoepithelioma like
    - Plasmacytoid/Signet ring cell/Diffuse
    - Sarcomatoid
    - Giant cell
    - Poorly differentiated
    - Lipid rich
    - Clear cell
- Non invasive urothelial neoplasms
    - Urothelial carcinoma in situ
    - Non invasive papillary urothelial carcinoma, low grade
    - Non invasive papillary urothelial carcinoma ,high grade
    - Papillary urothelial neoplasm of low malignant potential
    - Urothelial papilloma
    - Inverted urothelial papilloma
    - Urothelial proliferation of uncertain malignant potential
    - Urothelial dysplasia

Squamous cell neoplasms:

- Pure squamous cell carcinoma
- Verrucous carcinoma
- Squamous cell papilloma

Glandular neoplasms:

- Adenocarcinoma, NOS
    - Enteric
    - Mucinous
    - Mixed
- Villous adenoma

Urachal carcinoma

Tumours of Mullerian type:

- Clear cell carcinoma
- Endometrioid carcinoma

Neuroendocrine tumours:

- Small cell neuroendocrine carcinoma
- Large cell neuroendocrine carcinoma
- Well differentiated neuroendocrine tumour
- Paraganglioma

Melanocytic tumours:

- Malignant melanoma
- Naevus
- Melanosis

Mesenchymal tumours:

- Rhabdomyosarcoma
- Leiomyosarcoma
- Angiosarcoma
- Inflammatory myofibroblastic tumour
- Perivascular epithelioid cell tumour
    - Benign
    - Malignant

-Solitary fibrous tumour

- Leiomyoma
- Haemangioma
- Granular cell tumour
- Neurofibroma

Urothelial tract haematopoietic and lymphoid tumours:

Miscellaneous tumours:

- Carcinoma of Skene, Cowper and Littre glands
- Metastatic tumours and tumours extending from other organs
- Epithelial tumours of the upper urinary tract
- Tumours arising in a bladder diverticulam
- Urothelial tumour of the urethra

Histological typing of urothelial carcinoma:

Flat urothelial lesions:

Reactive urothelial atypia associated with acute and chronic inflammation. The inflammatory cell infiltrates the epithelium and lamina propria. Morphologically the

urothelium appear as basophilic with monomorphic elongated round nuclei, fine chromatin, no prominent nucleoli. Mitosis may frequent.

Urothelial carcinoma in situ is histologically heterogeneous. The urothelial cells are enlarged, more runded and disorganized with loss of polarity. The nucleus shows membrane irregularity with coarse chromatin and occasional nucleoli. Atypical mitosis may be seen.

Urothelial atypia of unknown significance and urothelial dysplasia very difficult to distinguish.They are put in single category because both diagnostic categories do not progress to higher grade lesion and have same clinical management.

Paillary urothelial neoplasms:

Urothelial papilloma:

Exophytic type:

It is composed of simple and occasional branching papillary architecture with fibrovascular core. They are lined by normal urothelium having prominent umbrella cells.

Inverted urothelial papilloma:

It is benign epithelial tumour with absence of papillae. The epithelium shows invagination and cells are arrange in cords and trabecular patter with peripheral palisading with central spindling. No cytological atypia and mitosis.

Papillary urothelial neoplasm of low malignant potential:

It is composed of delicate and fused papillae. The papillae lined by urothelial cells with increased thickness but they are maintaining the polarity with basement membrane. The cells are uniformly enlarged and round having fine nuclear chromatin, inconsipicuous nucleoli. Rare basal mitosis is seen.

Non invasive papillary urothelium carcinoma, low grade:

The papillae are branched and fused. The lining urothelium are cohesive and exhibit minimal crowding and loss of polarity. Mild variation in size and shape of the cells were present. Occasional mitosis is seen in any level. Umbrella cells usually present.

Non invasive papillary urothelial carcinoma, high grade:

They are characterized by fused, branched and delicate papillae with moderate to marked cytological atypia and nuclear hyperchromasia. Frequent mitosis with absent umbrella cell is common.

Invasive urothelial carcinoma:

The invasive carcinoma shows irregular nest of tumour cells that infiltrates the various layers of the bladder wall. Stromal response includes fibrosis, inflammatory reaction, desmoplasia, myxoid change and formation of retraction cleft. Invasive urothelial carcinoma shows divergent differentiation includes Invasive urothelial carcinoma with squamous differentiation or glandular differentiation and urothelial carcinoma with trophoblastic differentiation.

Other variants of urothelial carcinoma include microcystic, micropapillary, plasmacytoid, nested variant, lymphoepithelioma like carcinoma and sarcomatoid carcinoma.

Diagnosis:

The clinical history of the patients like history of cigarette smoking and occupational exposure to certain chemical are more important. Most of the patients present with symptoms like hematuria, frequency, urgency and abdominal pain.

Urine cytology:

Urine cytology helps to identify the exfoliated malignant cells in urine. It is a simple non invasive test for detection and monitoring the treatment response of BC. It has moderate sensitivity for detection of high grade tumours with more than 90% specificity. [80] In low grade tumour malignant cells are cohesive and have similar cytomorphology to normal urothelial cells so the sensitivity for detection is 20-50% with similar specificity. [81- 82]The major limitation of urine cytology is that the source of malignant cells could not be identified.

Cystoscopy:

Cystoscopy is an invasive procedure done under transurethral or intravesical topical anaesthesia .Clinically symptomatic patients are evaluated with cystoscopy to identify the presence of any lesion in bladder. It provides the information about the tumour site and size and also allows the collection of specimen or biopsy if any abnormality identified. Fluorescence cystoscopy is very helpful for detection of flat or

in situ lesion. Photosensitizer such as 5-aminolevulinic acid or hexaminolevulinic acid instilled intravesically and this allows the tumour tissue taking up the fluorescence to become macroscopically visible. [83]

Bladder wash cytology:

Urothelium appears grossly normal in situ lesion; bladder wash cytology detects almost all these lesions and avoids the need for random biopsies. [84]

Transurethral resection and biopsy:

Transurethral resection of bladder tissue (TURBT) is performed to remove the entire visible abnormality as both diagnostic and therapeutic procedure. During this procedure first superficial bulky tumour is removed followed by sampling of base of the tumour, which includes muscularis propia for appropriate staging of the tumour.

Urine markers:

Numerous marker are available for diagnosing the bladder cancer .These includes detection of mucin like protein and carcinoembronic antigen (ImmunoCyt test), Nuclear matrix protein -22(NMP-22 test), complement H related protein (Bladder tumour antigen – BTA test) and chromosomal abnormality(UroVysion test) etc. ImmunoCyt test have the high sensitivity (57-100%) and specificity (64-95%) than others. Sensitivity and specificity is depends on the tumour grade, stage and size.

Other investigation:

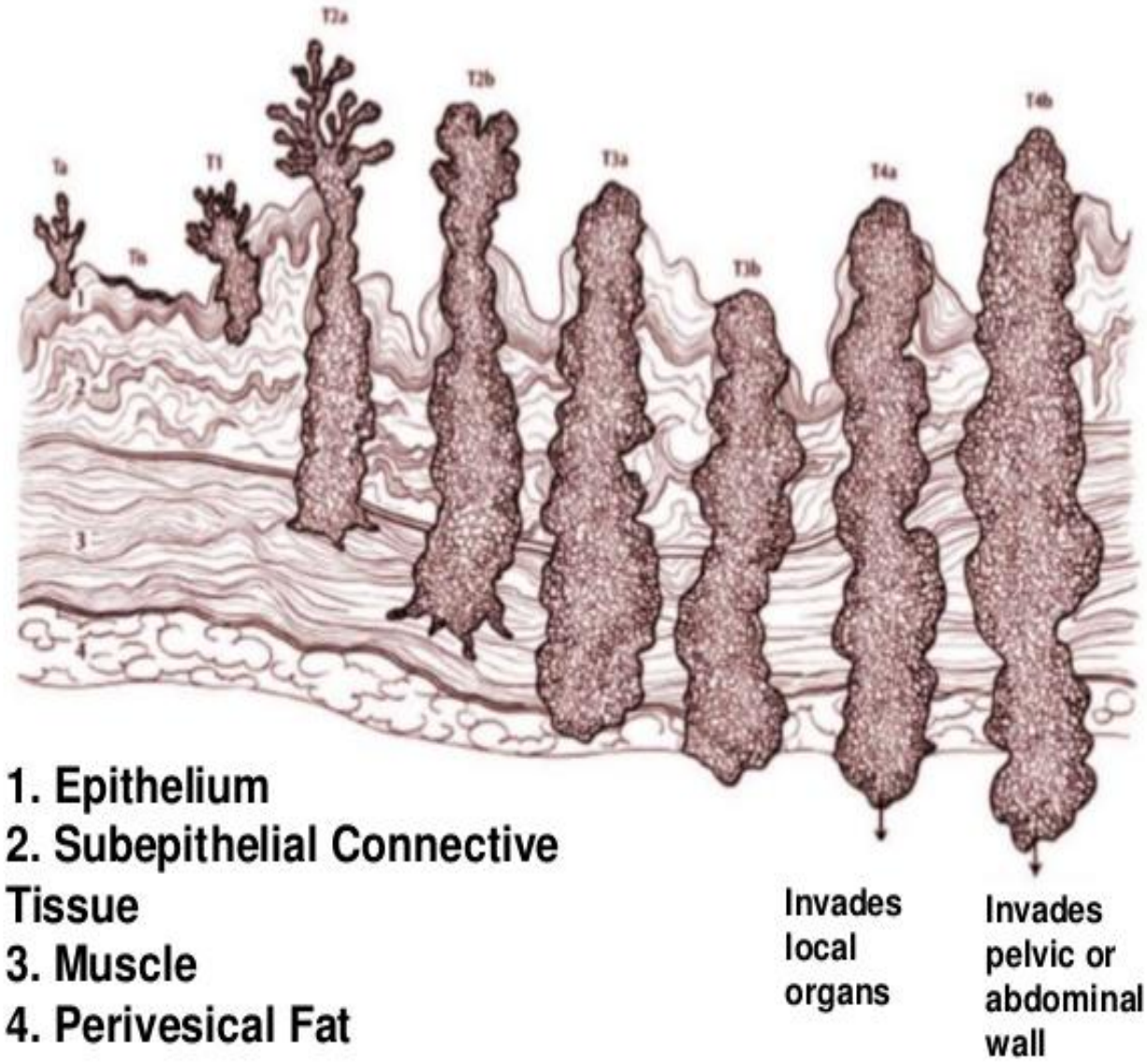
For evaluation of upper urinary tract additional work up needed for all bladder cancer patients. These include intravenous urography (IVU), renal ultrasonography, computed tomography (CT) urography, or magnetic resonance urography. [85- 86]

Staging and grading of bladder cancer:

Tumour size, Lymph node status and the presence of distant Metastasis are included in the pTNM system. [87] The staging of bladder cancer mainly depends on the depth of invasion and is the single most important determinant for patient survival.

Grading of urothelial carcinoma have prognostic significance and important particularly in non invasive lesions. Because most of the invasive carcinomas are in higher grade, only small proportion are low grade and limited to lamina propria. There is high inter observer variability in grading of papillary urothelial neoplasms, even among the experienced pathologists.

Figure: 7 Stages of urothelial carcinoma



BLADDER CANCER - PATHOLOGIC STAGE CLASSIFICATION (pTNM, AJCC 8TH EDITION)

TNM Descriptors:

___ m (multiple primary tumours)

___ r (recurrent)

___ y (post treatment)

Primary Tumor (pT):

___ pTX: Primary tumour cannot be assessed

___ pT0: No evidence of primary tumour

___ pTa: Non invasive papillary carcinoma

___ pTis: Urothelial carcinoma *in situ*: "flat tumour"

___ pT1: Tumour invades lamina propria (sub epithelial connective tissue)

___ pT2: Tumour invades muscularis propria

___ pT2a: Tumour invades superficial muscularis propria (inner half)

___ pT2b: Tumour invades deep muscularis propria (outer half)

___ pT3: Tumour invades perivesical soft tissue

___ pT3a: Tumour invades perivesical soft tissue microscopically

___ pT3b: Tumour invades perivesical soft tissue macroscopically (extravesical mass)

___ pT4: Extravesical tumour directly invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall

___ pT4a: Extravesical tumour invades directly into prostatic stroma, seminal vesicles, uterus, or vagina

___ pT4b: Extravesical tumour invades pelvic wall, abdominal wall

Regional Lymph Nodes (pN) :

___ pNX: Lymph nodes cannot be assessed

___ pN0: No lymph node metastasis

___ pN1: Single regional lymph node metastasis in the true pelvis (perivesical, obturator, internal and external iliac or sacral lymph node)

___ pN2: Multiple regional lymph node metastasis in the true pelvis (perivesical, obturator, internal and external iliac or sacral lymph node metastasis)

___ pN3: Lymph node metastasis to the common iliac lymph nodes

Distant Metastasis (pM) :

___ pM1: Distant metastasis

___ pM1a: Distant metastasis limited to lymph nodes beyond the common iliacs

___ pM1b: Non-lymph node distant metastases

Management:

Treatment of bladder cancer depending on the age of the patient, surgical risk, the extent, stage and microscopic grade of the tumour and presence of in situ lesion elsewhere in the bladder.

TURBT without intravesical chemotherapy is used for low grade T_a tumours. For preventing the recurrence a single dose of intravesical chemotherapy within 24 hours of resection also suggested. [88]

In case of high grade T_a tumour with presence of lymphovascular invasion or incomplete resection or no muscle in the specimen, a repeat the resection followed by intravesical BCG or mitomycin may be required.

Carcinoma in situ, both low and high grade T_1 tumours are treated like high grade T_a tumour.

T_2 tumour is treated by radical cystectomy followed by chemotherapy in high-risk patients (presence of nodal involvement, high grade histology, transmural or vascular invasion).

For T_3 tumour radical cystectomy with neoadjuvant chemotherapy has shown survival benefit (three cycles of methotrexate, vinblastine, doxorubicin [Adriamycin], and cisplatin [Platinol]).

T4 or metastatic disease treated with chemotherapy alone or in combination with radiation therapy. An emerging immune check point inhibitor (anti PDL1) shows promise effect in patients with locally advanced or metastatic urothelial carcinoma who have disease progression during or following platinum-containing chemotherapy. [89]

Prognostic factor in bladder carcinoma:

To predict the recurrence and progression of bladder cancer the prognostic factors such as primary or recurrent tumour, type of tumour, number and size of tumour, grade and stage of tumour and presence of in situ lesion are helpful .[90- 91].

p63:

p63 is a nuclear transcription factor belongs to p53 group of family encoded by a gene located on chromosome 3q 27-29. [92] Normal proliferation of basal cells in urothelium depending on the p63 which act as myoepithelial cell marker. [93] It consisting of 15 exons and two promoters. Upstream transcription from the first promoter to exon 1 gives full length protein called TAp63. Similar transcription from

second promoter leads to production of N- terminally truncated protein isoforms, ΔNp63. Several C-terminal protein isoforms, like α, β, γ and δ, ε are formed due to alternative splicing of the 3' end of TP63 mRNA. This explains p63 protein diversity**.**

p63 especially TAp63 isoform act as a tumour suppressor and its over expression leading to cell cycle arrest and mediate apoptosis by triggering death receptor complexes (CD95,TRAIL) and mitochondrial death pathway (BAX, APAF1). [(94)]

ΔNp63 act as oncogene thus inhibits death receptors mediated apoptosis and chemotherapy induced mitochondrial apoptosis pathways. [(95)]

Few study proposed that over expression of p63 mRNA relates to carcinogenesis and tumour progression. In contrast to other studies shows high-grade invasive urothelial carcinomas frequently diminish p63 expression, whereas low-grade tumours highly preserve the normal p63 expression. [(96)]

Impaired p63 expression is thought to be a prognostic marker with the well-established prognostic factors, such as TNM stage, indicating that impaired p63 characterizes biological aggressiveness of urothelial neoplasms. Recently p63 has been shown to be a marker of tumours of urothelial origin**.**

Expression of p63 expression in various studies:

S.NO	Studies	Papilloma	PUNLMP	Papillary UC low grade	Papillary UC high grade
1.	Ali koyuncuer et al (2016)	-	-	85% shows increased expression	27% shows decreased expression
2.	Elnashar et al (2016)	-	-	94% shows increased expression	72% shows decreased expression
3.	Sayed abdul rahem et al (2014)	-	-	90% shows increased expression	65% shows decreased expression
4.	Dimitra graspa et al (2014)	-	-	57% shows increased expression	63% shows decreased expression

Ki-67:

Ki-67 is a non histone nuclear protein encoded by a gene present in chromosome 10q26.2. It contains many functional regions include an N-terminal fork head-associated (FHA) domain, a protein Phosphatase 1 (PP1) binding domain, a large central region comprising tandem repeats, and a C-terminal LR (leucine/

arginine-rich) chromatin-binding domain .All variants of Ki-67 have identical central tandem repeats and C terminal region. [97- 98]

Different isoforms of Ki-67 shows various impacts on cell proliferation and progression of cell cycle. Two protein isoforms with molecular weights of 320 kDa and 350 kDa were encoded by the two major transcript variants which differ by the alternative inclusion of exon7. Over expression of alternative exon7 reduce the proliferation, whereas over expression of an N-terminal fragment increases the cell proliferation. Expression of exon 7 causes translocation of cyclin B from the cytoplasm to the nucleolus and leads to mitosis initiation. Mitotic localization of Ki-67 is regulated by CDK/cyclin phosphorylation and PP1 dephosphorylation. [99] Ki-67 is a major nuclear structural transition during mitotic entry and exit.

It is expressed in all phases of cell cycle except G0 and act as a marker of cellular proliferation. Cellular content of Ki-67 protein markedly increases during cell progression through S phase of cell cycle. [100]

Expression of Ki-67 expression in various studies:

S.NO	Studies	Papilloma	PUNLMP	Papillary UC low grade	Papillary UC high grade
1.	Noora Ali Jawad et al (2016)	-	-	71% shows decreased proliferation	40% shows increased proliferation
2.	Vitaly margulius et al (2006)	-	-	82% shows decreased proliferation	45% shows increased proliferation
3.	Cina et al (2001)	1-8%	0.5-15%	0.5-38.5%	1-65%
4.	Dipli Grajjar et al (2014)	1-6%	2-10%	15-40%	-
5.	Quintero et al (2004)	100% ,< 13% expression	96.2%,<13% expression	88.2%,<13% expression	73.3%, <13% expression

MATERIALS AND METHODS

MATERIALS AND METHODS

Study design:

This is a laboratory-based prospective and retrospective study.

Study period:

This study was carried out between April 2018 and September 2019.

Place of study:

The study was conducted in the Department of Pathology at Dhanalakshmi Srinivasan Medical College and Hospital (DSMCH).

Study population:

The study population included cases with urothelial carcinoma reported in the pathology department of DSMCH during the period of July 2015 to June 2019.

Sample size:

50 cases of primary urothelial carcinoma.

Inclusion criteria:

1. Cystoscopy guided biopsy, transurethral resection of bladder tissue and cystectomy specimens diagnosed as urothelial neoplasm.
2. Sufficient archival tissue in formalin fixed , paraffin embedded blocks to perform analysis

Exclusion criteria:

1. Benign urothelial lesions
2. Non urothelial carcinoma

3. Recurrent tumour
4. Insufficient tissue blocks
5. Unsatisfactory or equivocal immunostaining due to technical factors

The study was commenced after obtaining a letter of approval by the Institutional ethics committee, DSMCH.

Primary urothelial carcinoma cases which satisfied the selection criteria were taken from the department archives. The clinical details of the patients including age, sex, procedure were obtained from the documents.

Corresponding H&E slides and paraffin blocks were retrieved. The diagnosis and necessary histopathological parameters were reviewed. Ki-67 and p63 IHC were performed for all cases.

Immunohistochemical evaluation:

PolyExcel Horse Radish peroxidase poymer (HRP) / Diamino benzidine substrate (DAP) was used for analyse the Ki-67 and P63 (pathnsitu) immunohistochemistry. From formalin fixed paraffin embedded tissue blocks, 4 micron tissue sections were cut then tissue section was transferred onto positively charged slides. Heat induced antigen retrieval was done. The antigen was bound with mouse monoclonal antibody against Ki-67, P63 (pathnsitu) protein and then detected by adding secondary antibody conjugated with HRP/DAB substrate.

Evaluation of Ki-67 labeling index:

The percentage of ki-67 index was calculated by counting at least 500 cells in high power fields in areas of maximum nuclear positivity. Nuclear positivity was seen

as dark brown colour on bluish background. The results were consider low proliferation (<20% expression) and high proliferation (>20% expression). [101]

p63 scoring:

For assessing the p63 immunostaining at least 500 cells were evaluated after selecting high-power (400x) fields. Immunohistochemical staining was scored according to previously validated protocols. Staining for p63 was considered increased or decreased expression when >10% or <10% of tumour cells showed strong nuclear staining respectively. [102]

Antigen	Clone	Positive control	Source	Working dilution
Ki-67	GM001	Tonsil	Mouse monoclonal	Ready to use
P63	4A4	Prostate	Mouse monoclonal	Ready to use

Table: Criteria for Ki-67 and p63 expression

Marker	Localization	Expression pattern	
Ki- 67	Nuclear	Less than 20% of stained tumour cells –low proliferation	More than 20% of stained tumour cells -high proliferation
p63	Nuclear	Less than 10% of stained tumour cells – decreased expression	More than 10% of stained tumour cells –increased expression

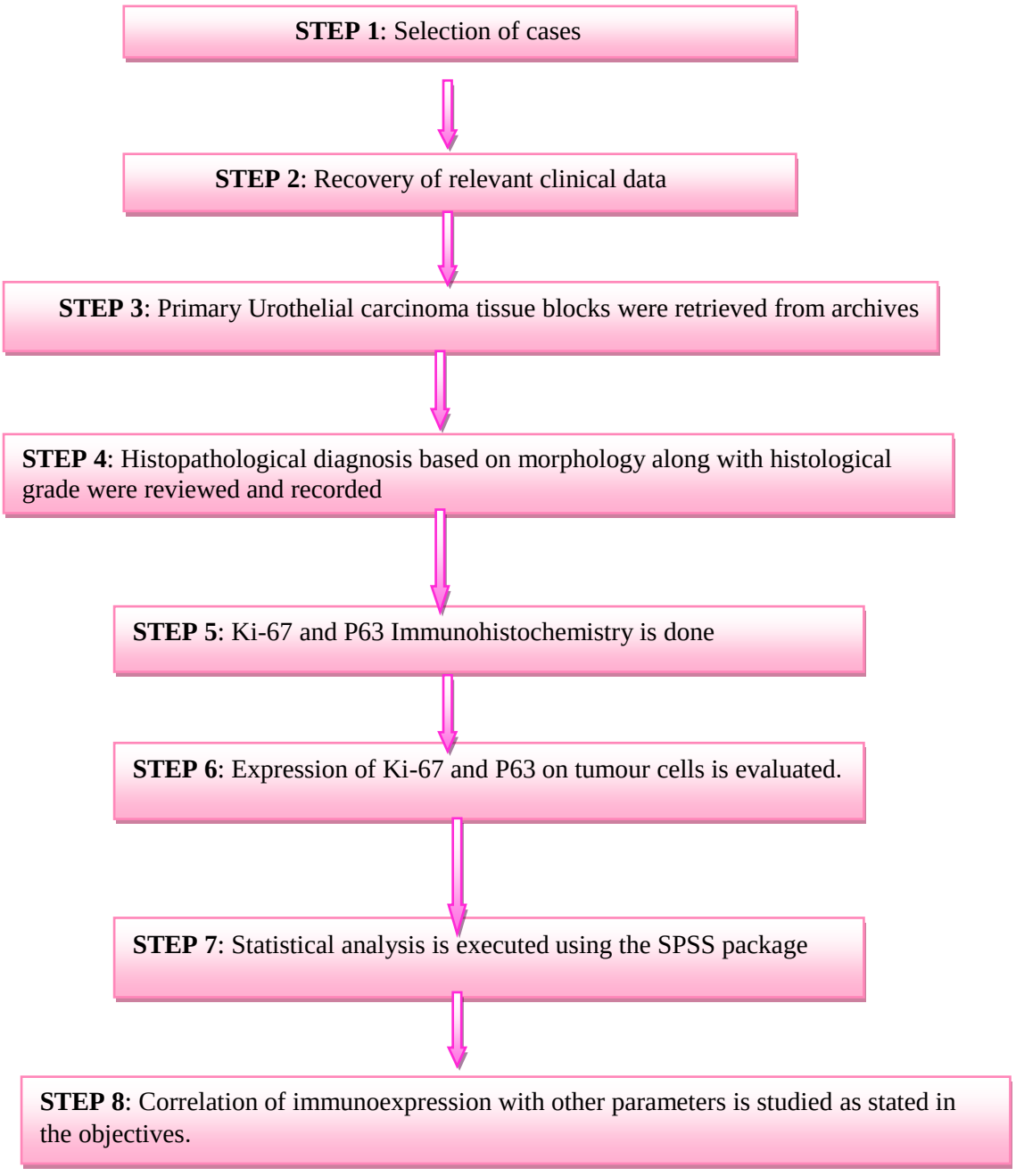
Statistical analysis:

Collected data was filled in the master chart. Master chart data's were entered into the computer then data was verified and coded. Initial analyses of collected variables were performed. Correlation of IHC expression of Ki-67 and p63 with other

parameters like age, gender, grade and stage were done. The data was analyzed by using SPSS (Statistical Package for Social Sciences) computer version 21.0 and Microsoft Excel 2013. P value below 0.05 considered significant.

To assess the difference between the two parameters Chi square test was used for qualitative variables. Data is presented using bar graphs, pie charts and tables.

Algorithm:



RESULTS

RESULTS

During the period of four years from July 2015-June 2019, a total of 63 bladder biopsies/ specimens were received in the Department of Pathology, Dhanalakshmi Srinivasan Medical College and hospital for histopathological examination.

Out of 63 cases 60 were TURBT and 3 radical cystectomy specimens. Among 63 cases, 50 were reported as urothelial carcinoma (31 high grade and 19 low grade), 9 cases were reported as non urothelial carcinoma, 1 case was reported as urothelial dysplasia and 3 cases were non diagnostic due to scanty tissue in 1 biopsy and 2 biopsy specimens show only blood clot.

Among 9 cases of non urothelial carcinoma, 1 case was reported as granular cell tumour, 1 case diagnosed as mucinous carcinoma, 3 cases were poorly differentiated carcinoma, 4 cases were squamous cell carcinoma.

In this study we included 50 cases of histopathologically diagnosed urothelial carcinoma.

Chart: 4 Type of bladder specimens

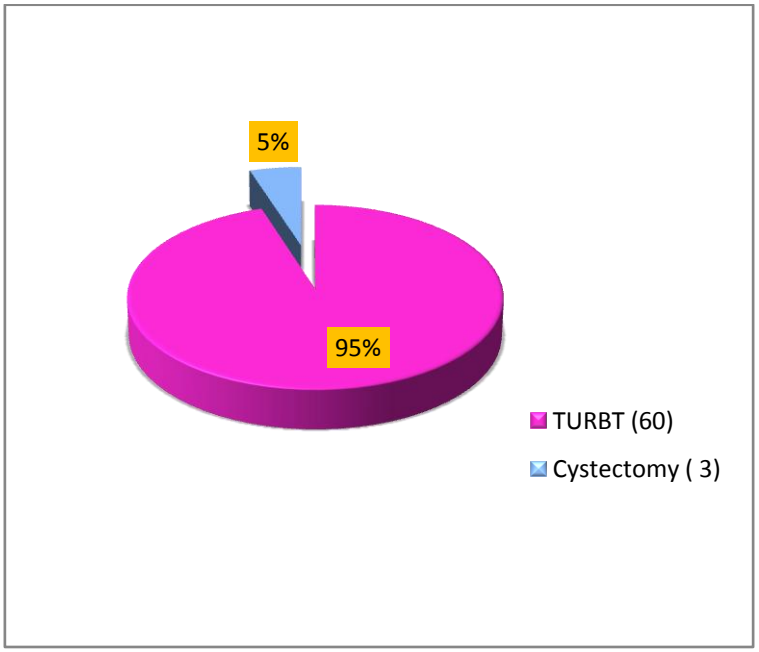


Table: 1 Distribution of diagnoses

Diagnosis	Total number
Urothelial carcinoma	50
Squamous cell carcinoma	4
Granular cell tumour	1
Mucinous carcinoma	1
Poorly differentiated carcinoma	3
Urothelial dysplasia	1

Among 50 cases of urothelial carcinoma, most of the cases were males (31 cases, 62%), females were 19 cases (38%) accounting to male to female ratio of 1.6:1 in this study.

A maximum number of cases were seen in the age group of above 60 years (29 cases, 58%) followed by age group 40 -60 years (19 cases, 38%), 2 cases (4%) in the age group of 20 -40 years. No case was reported below 20 years of age. The median age of presentation was 62 years.

Majority of the male cases (20 cases) and female cases (9 cases) were reported in the age group above 60 years. The minimum age of diagnosed case was 27 years (female) and maximum age of diagnosed case was 84 years (male) patient.

Chart: 5 Gender wise distribution of cases

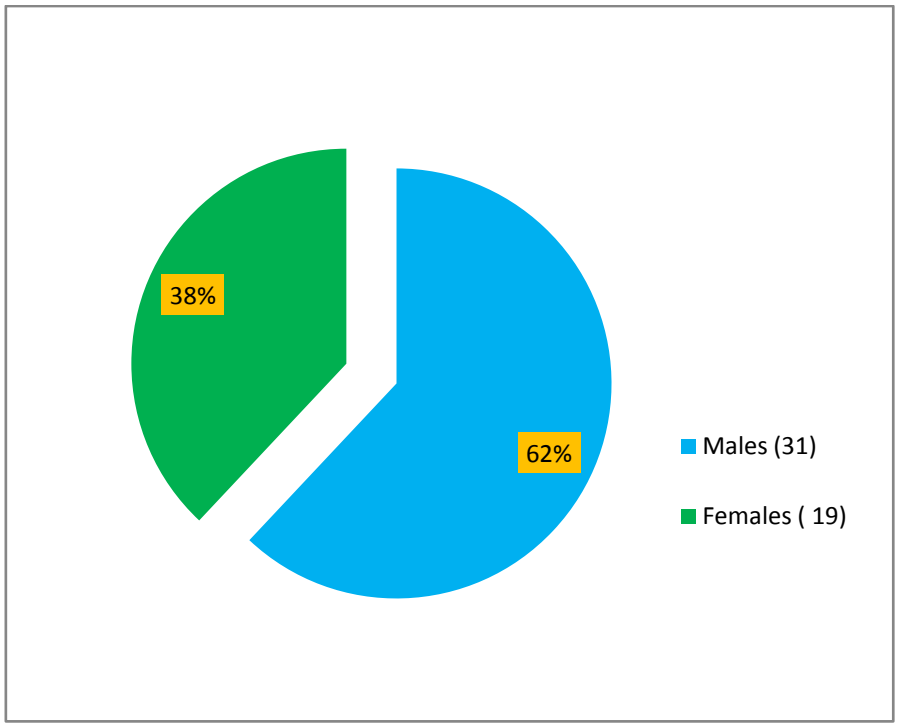


Chart: 6 Grade wise distribution of cases

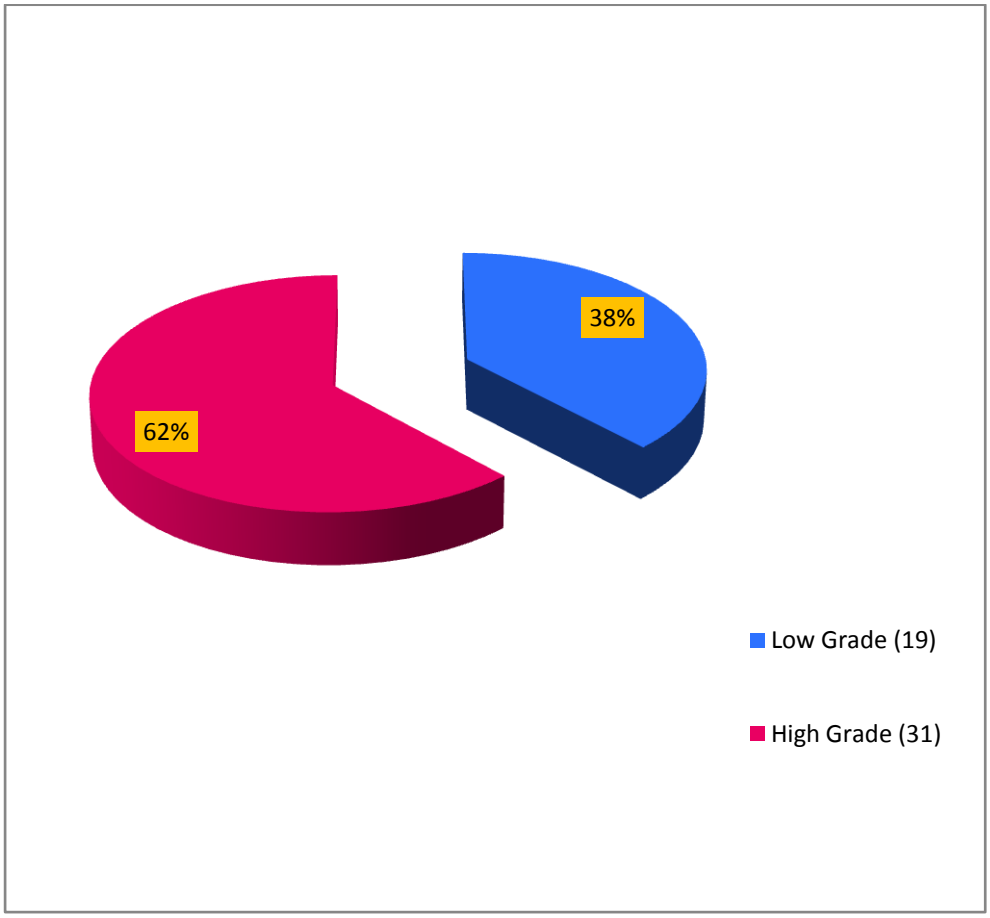
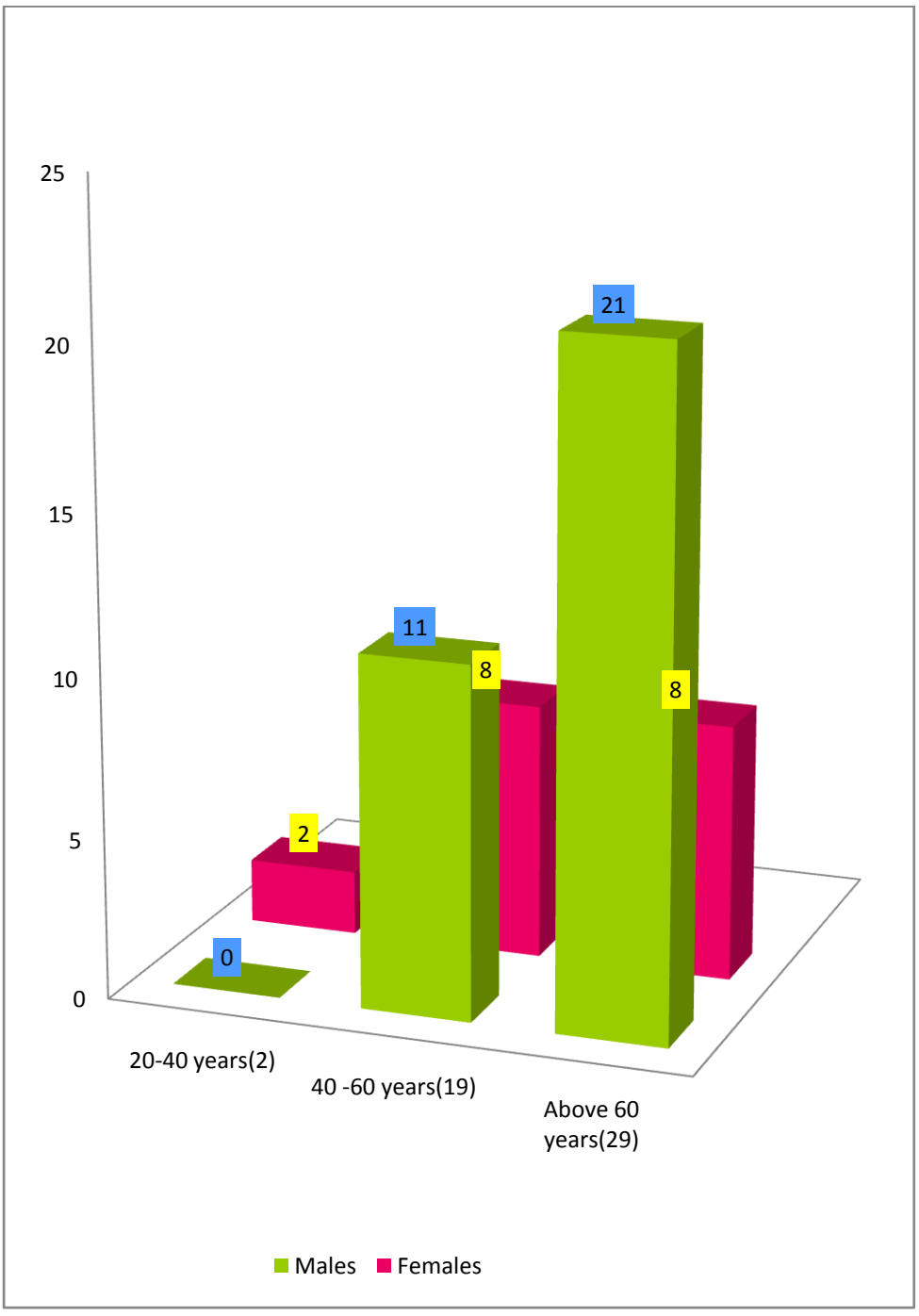


Chart: 7 Age group and gender wise distribution of cases



This study constituted 62% of males (31 cases of males, 13 cases were reported as low grade and 18 cases were high grade) and 42% of females (19 cases of females, 6 cases were low grade and 13 cases were high grade)

Chart: 8 Gender wise distribution of varying grades urothelial carcinoma

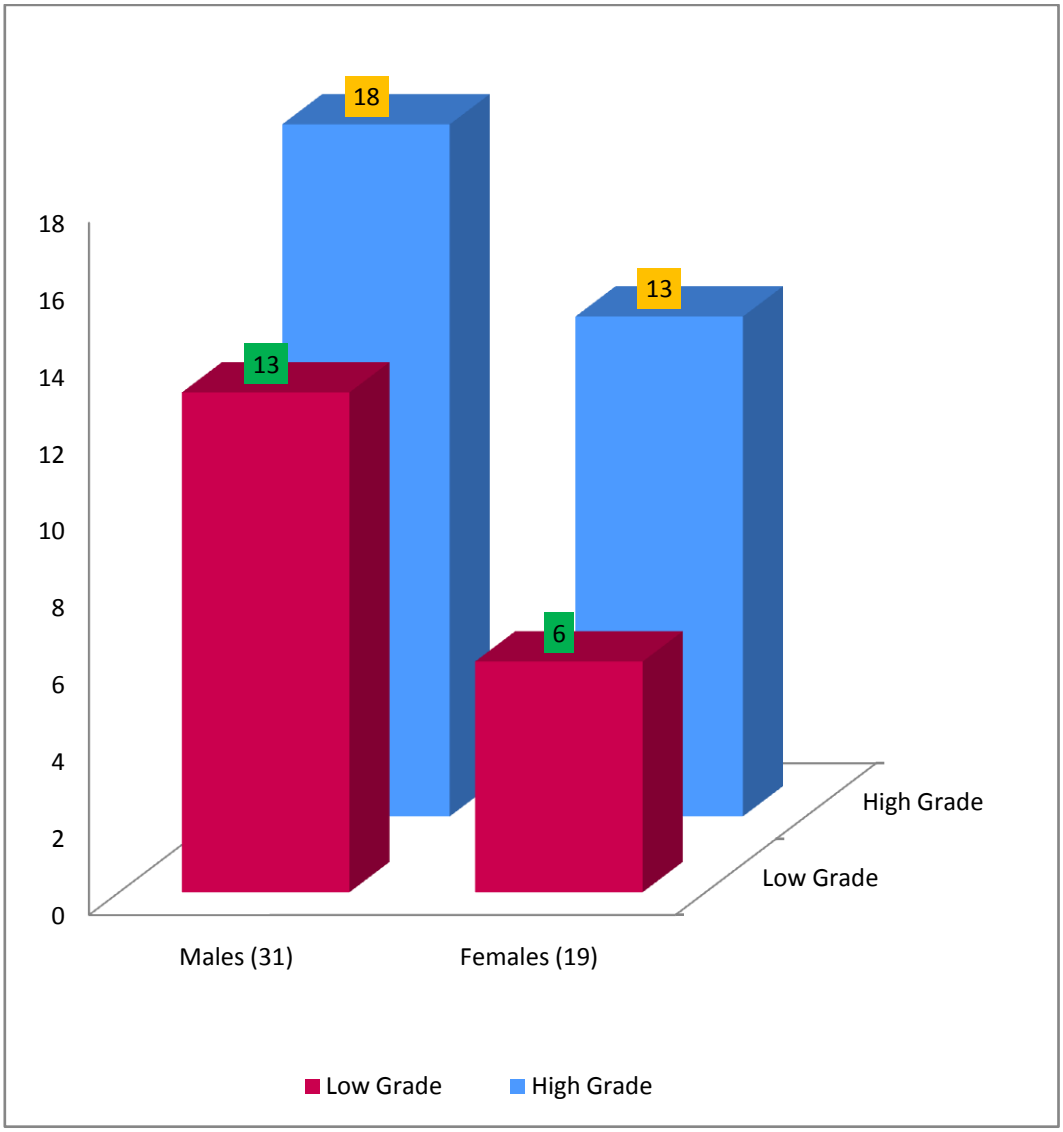


Table: 2 Correlation of histological grade with gender

Histological grade	**Gender**				**Total**		**Statistical inference**
	Male		Female				
	n	%	n	%	N	%	
Low grade	13	41.9	6	31.6	19	38	X^2 =0.536 P value =0.464
High grade	18	58.1	13	68.4	31	62	

Out of 19 cases of low grade urothelial carcinoma, 13 cases (41.9%) were males and 6 cases were (31.6%) females. In 31 cases of high grade urothelial carcinoma, 18 case were males (58.1%) and 13 cases were (68.4%) females.

Even though incidence of urothelial carcinoma is lower in females than males, females were presented with higher grade of the tumour. There was no statistically significant correlation between gender and histological grade of the urothelial carcinoma.

Chart: 9 Distribution of varying grades of urothelial carcinoma among various age groups in male patients.

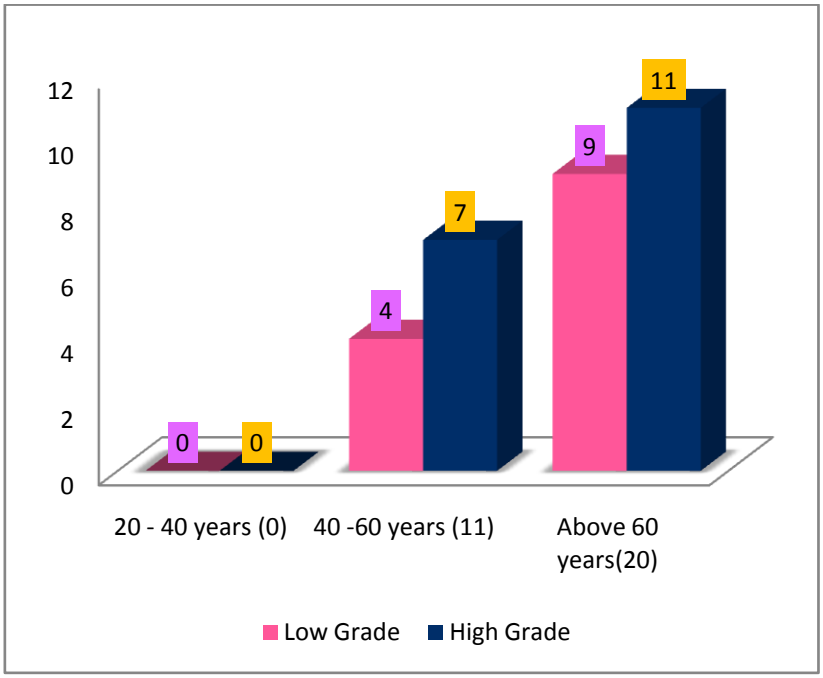


Chart: 10 Distribution of varying grades of urothelial carcinoma among various age groups in female patients.

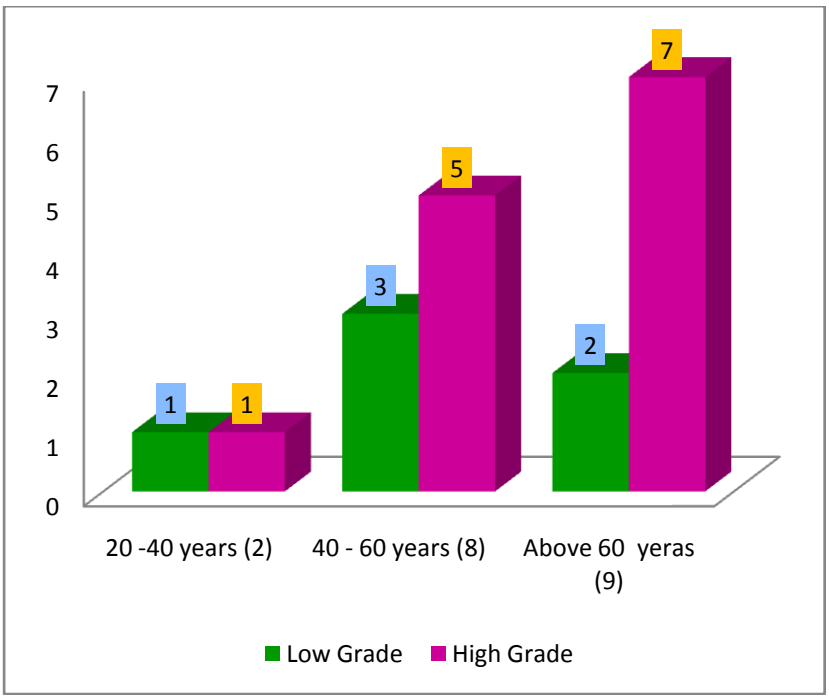


Table: 3 Correlation of histological grade with varying age groups

Age group	Histological grade				Total		Statistical inference
	Low grade		High grade				
	n	%	n	%	n	%	
20 - 40 years	1	5.3	1	3.2	2	4	X^2 =0.133 P value =0.932
40 – 60 years	7	36.8	12	38.7	19	38	
Above 60 years	11	57.9	18	58.1	29	58	

Majority of the low grade and high grade urothelial carcinomas were in the age group of above 60 years and accounting to 57.9% (11 cases), 58.1% (18 cases) respectively. This observation was statistically not significant. So there is no association between the histological grade of the urothelial carcinoma and age.

Among 50 specimens diagnosed as urothelial carcinoma , TURBT specimens were 47 cases and 3 cases were cystectomy specimen. Out of 50 specimens, muscle tissue was included in biopsy is 22 cases. Remaining 28 cases did not have muscle tissue in the specimens.

Chart: 11 Presence of muscle tissue in biopsies

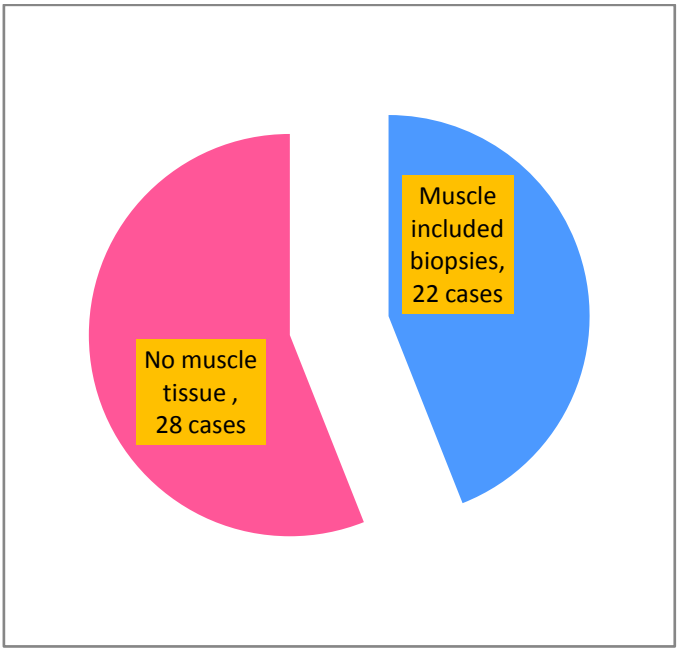
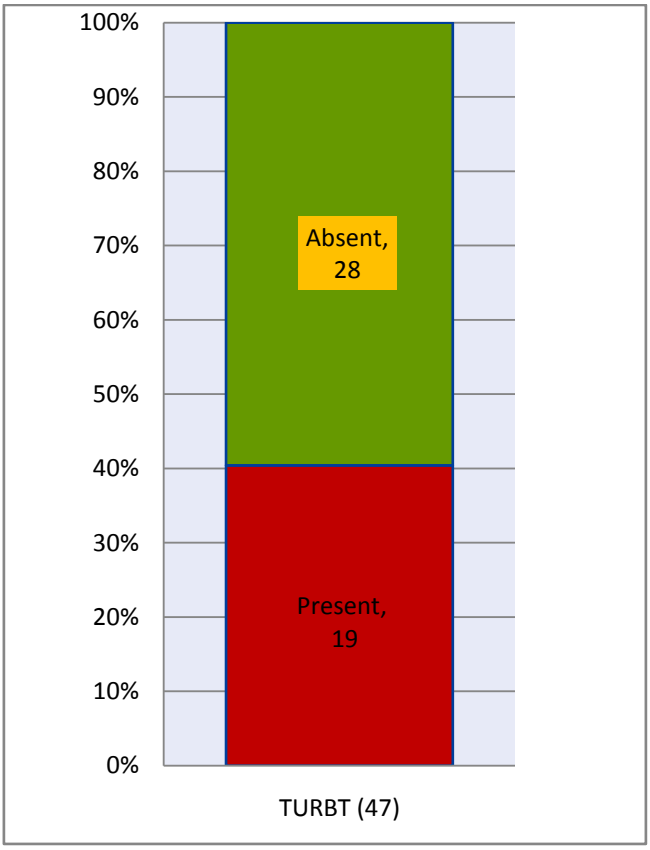


Chart: 12 Presence of muscle tissue in TURBT specimens



Among 19 cases of TURBT, 13 cases shows muscle invasion and 6 cases were non muscle invasive urothelial carcinoma. In 3 cases of cystectomy specimens 2 cases showed muscle invasion whereas 1 case was non muscle invasive urothelial carcinoma.

Chart: 13 Presence are absence of muscle invasion

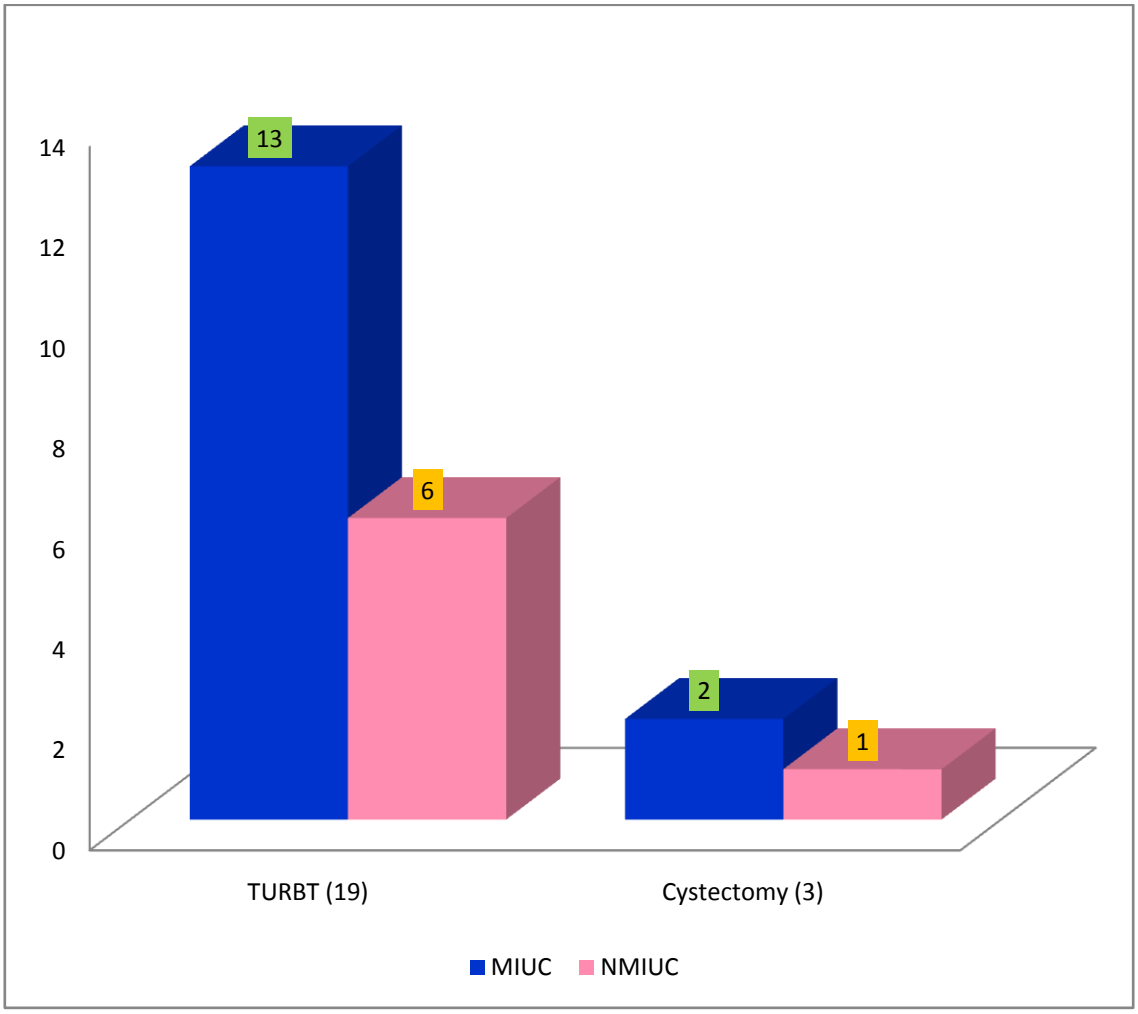
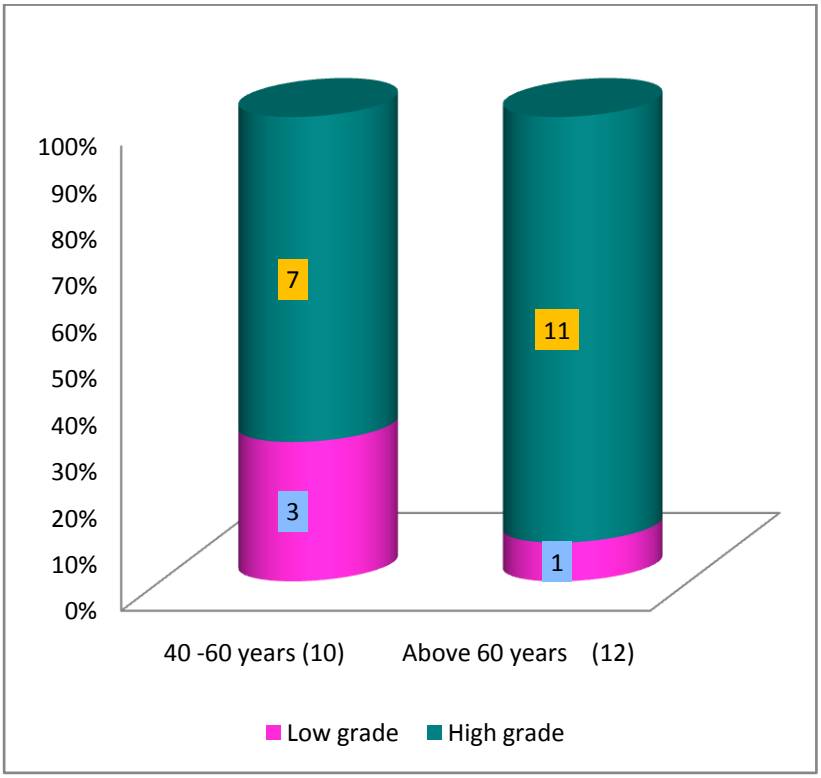


Table: 4 Distribution of low grade and high grade urothelial carcinoma among the varying age groups and status of muscle invasion

Age Group		Low Grade		High Grade	
		Muscle Invasion		Muscle invasion	
		Present	Absent	Present	Absent
40 -60 years (10)	Males (6)	0	1	3	2
	Females (4)	1	1	2	0
Above 60 years (12)	Males (7)	1	1	4	**2**
	Females (5)	0	0	5	0

Chart: 14 Distribution of low grade and high grade urothelial carcinoma among varying age groups



In this study out 50 cases, 22 specimens were had muscle tissue in biopsy/cystectomy. Out of this 10 cases were in the age group of 40 -60 years and 12 cases were in the age group of above 60 years. Among this males were 13 cases (2 cases were low grade, 11 cases were high grade) and females were 9 cases (2 cases were low grade, 7 cases were high grade).

According to the invasion of the muscle tissue 15 cases were grouped in the stage of $\geq T_2$ and above the stage, 7 cases did not show muscle invasion so there were grouped in the stage of T_a or T_1

Chart: 15 Gender wise distribution of cases

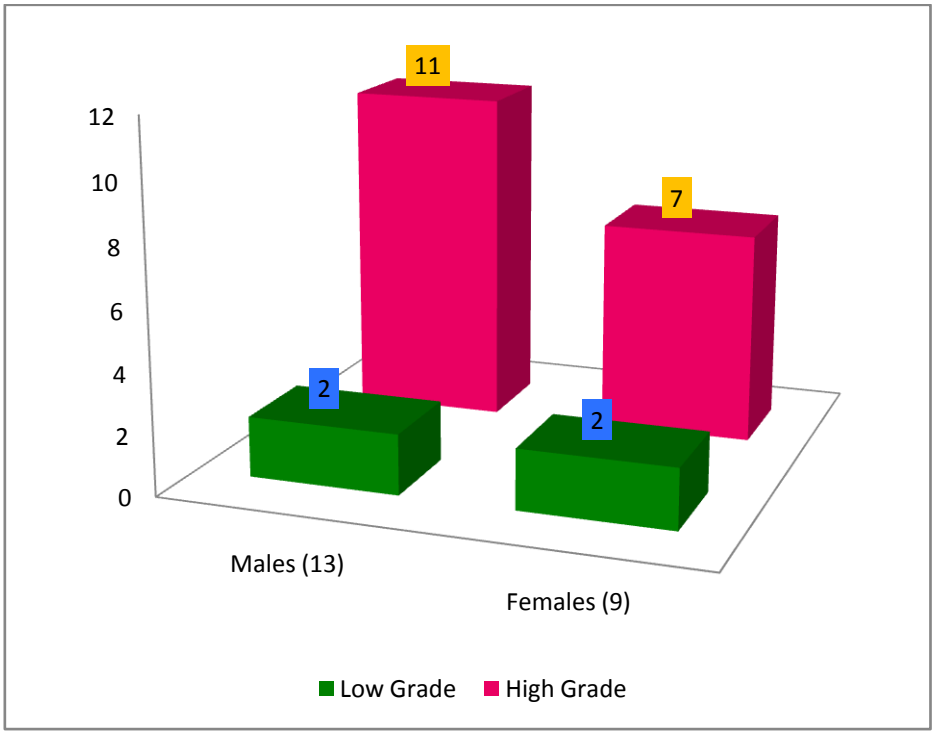


Chart: 16 Distribution of cases in varying grades among various stages

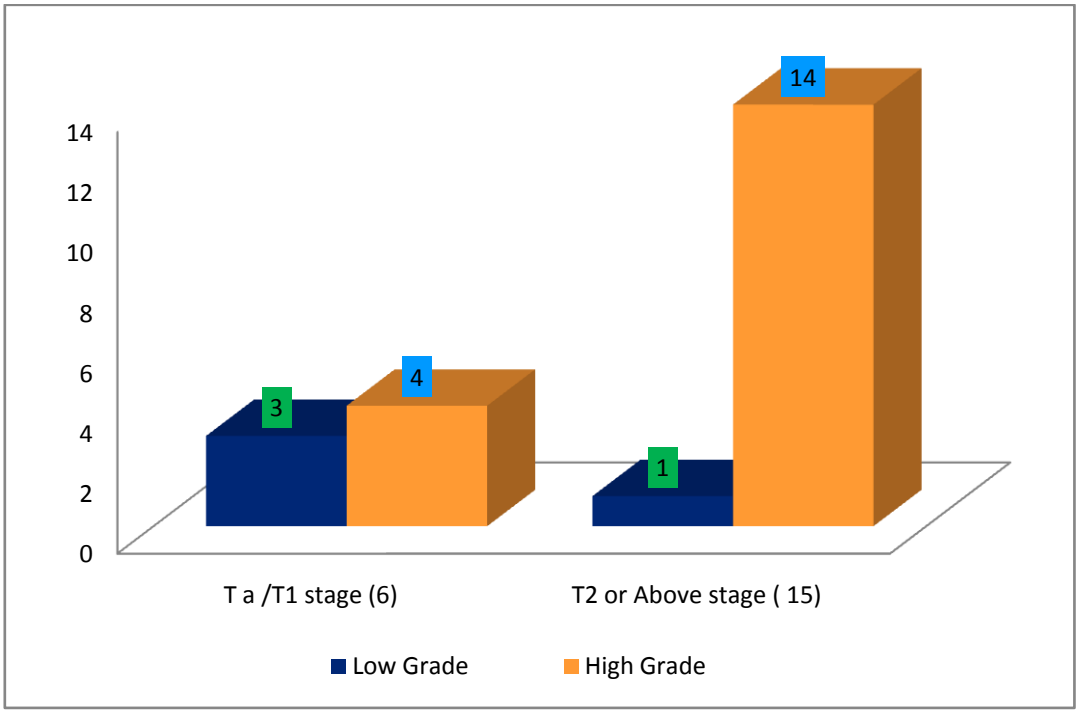


Chart: 17 Gender wise distribution of cases among various stages

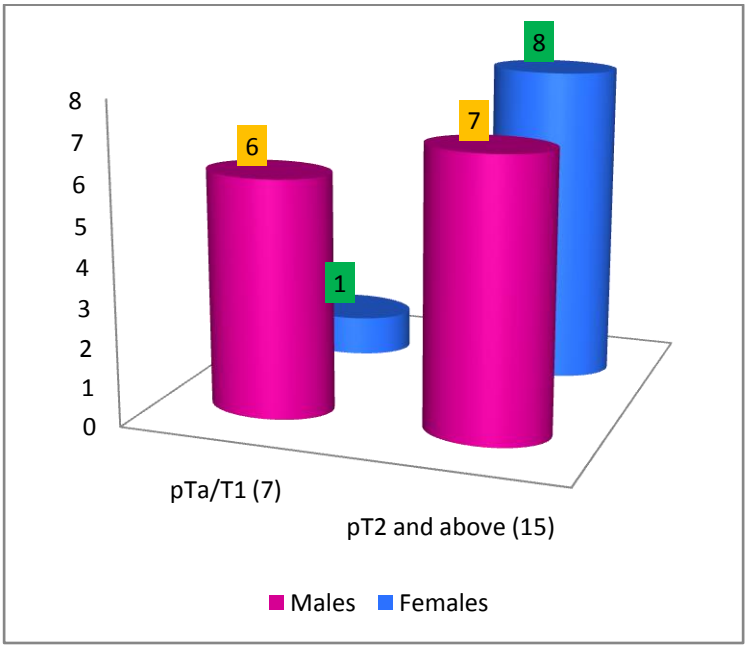
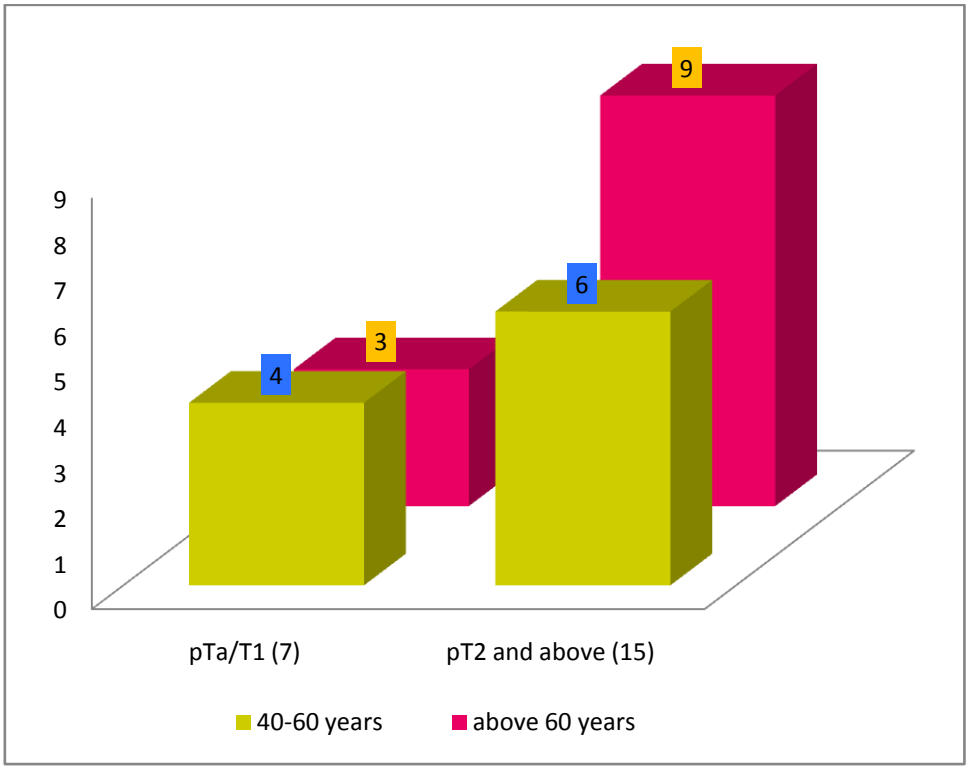


Chart: 18 Age wise distribution of cases among various stages



Expression of Ki-67:

The cut off for increased expression of Ki-67 was nuclear positivity in more than 20% of the tumour cells and the expression less than 20% were considered decreased and these criterias have been followed throughout the Ki-67 evaluation.

Table: 5 Correlation of Ki-67 expression with age

Age group	**< 20% expression**		**>20% expression**		**Total**		**Statistical inference**
	n	%	n	%	n	%	
20 – 40 years	1	5	1	3.3	2	4	X^2 =0.170 P value =0.918
40 -60 years	8	40	11	36.7	19	38	
Above 60 years	11	55	18	60	26	58	

Among 50 cases of urothelial carcinoma, 20 cases shows < 20% of expression of Ki-67 index. Remaining 30 cases were shows > 20% of Ki-67 expression. Most of the cases above 60 years show higher percentage of both increased and decreased expression of Ki-67 index 55% (n =11 cases) and 60% (n = 18 cases respectively).

This observation does not show any statistically significant correlation among Ki-67 expression with varying age groups. The p value was above 0.05.

Table: 6 Correlation of Ki-67 expression with gender

Gender	**< 20% expression**		**>20% expression**		**Total**		**Statistical Inference**
	N	%	n	%	n	%	
Male	14	70	17	56.7	31	62	X^2 =0.905
Female	6	30	13	43.3	19	38	P value = 0.341

Out of 20 cases of male, 14 cases (70%) shows decreased (<20%) expression of Ki-67 and 17 cases (56.7%) shows increased (>20%) expression of ki-67 labeling index. In 19 cases of females ,6 cases (30%) shows decreased expression of Ki -67 and 13 cases (43.3%) shows increased expression of Ki-67 labeling index.

This study shows no statistically significant correlation between the Ki-67 expression with gender. P value was 0.341.

Table: 7 Correlation of Ki-67 expression with histological grade

Grade	< 20% expression		> 20% expression		Total		Statistical inference
	n	%	n	%	n	%	
Low grade	16	80	3	10	19	38	X^2 =24.958
High grade	4	20	27	90	31	62	P value =0.000

Among 20 cases of decreased (<20%) expression of Ki – 67 labeling index, 16 cases (80%) were low grade and 4 cases (20%) were high grade. Out of 30 cases of increased (>20%) expression of Ki -67 labeling index 3cases (10%) were low grade and 27 cases (90%) were high grade.

As grade advances the expression of Ki -67 also increases. This observation was statistically significant and p value was less than 0.05.

This study shows significant association between Ki-67 expression and histological grade of urothelial carcinoma.

Table: 8 Correlation of Ki-67 expression with stage of tumour

Ki-67 expression	**Stage of tumour**						**Statistical inference**
	$T_{a\,/}\,T_1$		$T_{2\,\&}$ Above		Total		
	n	%	n	%	n	%	
<20%	3	42.9	2	13.3	5	22.7	X^2 =2.369
>20%	4	57.1	13	86.7	17	77.3	P value =0.124

Among 22 cases of muscle tissue included biopsies/specimens, 7 cases were diagnosed as stage of $pT_{a\,/}T_1$. In this 7 cases, 3 cases (42.9%) shows decreased (<20%) expression of Ki- 67 and 4 cases (57.1%) cases were shows increased (>20%) expression of Ki -67 labeling index.

Out of 22 cases, 15 cases were diagnosed as stage of pT_2 or above in stage. Among 15 cases, 2 cases (13.3%) shows decreased (<20%) expression of Ki -67 and 13 cases (86.7%) shows increased (>20%) expression of Ki – 67 labeling index.

Most of the cases in both stages of tumour shows increased expression of Ki -67 labeling index. This result shows p value of 0.124 and these was no statistically significant correlation of Ki -67 expression with invasive nature of the tumour.

Expression of p63:

The cut off for p63 expression was 10% of nuclear positivity and more than that considered increased expression and less than that was considered decreased expression. These criterias have been taken throughout the evaluation of p63 expression.

Table: 9 Correlation of p63 expression with age

S.NO	Age groups	<10% expression		>10% expression		Total		Statistical inference
		n	%	n	%	n	%	
1.	20-40 years	0	0	2	7.1	2	4	X^2 =2.227 P value =0.328
2.	40-60 years	10	45.5	9	32.1	19	38	
3.	Above 60 years	12	54.5	17	60.7	29	58	

Among 22 cases of decreased (<10%) expression of p63, 10 cases (45.5%) were in the age group of 40 -60 years, 12 cases (54.5%) were in the age group of above 60 years. None of the case in the age group of 20 -40 years shows decreased expression of p63.

Out of 28 cases of increased (>10%) expression of p63, 2 cases (7.1%) were in the age group of 20 -40 years, 9 cases (32.1%) were in the age group of 40 -60 years and 17 cases (60.7%) were in the age group of above 60 years.

Majority of the cases above 60 years shows increased or decreased expression of p63. The expression of p63 does not show statistically significant correlation with age. P value was above 0.05.

Table: 10 Correlation of p63 expression with gender

Gender	<10% expression		>10% expression		Total		Statistical inference
	n	%	n	%	n	%	
Male	14	63.6	17	60.7	31	62	X^2 =0.045
Female	8	36.4	11	39.3	19	38	P value =0.833

Among 22 cases of decreased (<10%) expression of p63, males were 14 cases (63.6%) and females were 8 cases (36.4%).

Out of 38 cases of increased (>10%) expression of p63, males were 17 cases (60.7%) and females were 11 cases (39.3%).

Most of the male cases show increased or decreased expression of p63. This result was statically not significant with p value of above 0.05. p63 expression occurred independently with gender.

Table: 11 Correlation of p63 expression with histological grade

Grade	< 10% expression		>10% expression		Total		Statistical inference
	n	%	n	%	N	%	
Low grade	1	4.5	18	64.3	19	38	X^2 =18.662
High grade	21	95.5	10	35.7	31	62	P value =0.000

Among 22 cases of decreased (<10%) expression of p63, 1 case (4.5%) was low grade urothelial carcinoma and 21 cases (95.5%) were high grade urothelial carcinoma.

Out of 28 cases of increased (>10%) expression of p63, 18 cases (64.3%) were low grad e and 10 cases (35.7%) were high grade urothelial carcinoma.

Majority of the low grade urothelial carcinoma shows increased expression of p63 whereas high grade urothelial carcinoma shows decreased expression of p63.

As advances the grade of the urothelial carcinoma the expression of p63 was decreased. This shows statistically significant correlation among the p63 expression and histological grade of the tumour. The p value was less than 0.05.

Table: 12 Correlation of p63 expression with stage of tumour

P63 expression	Stage of tumour						Statistical Inference
	T_a / T_1		T_2 & Above		Total		
	n	%	n	%	n	%	
<10%	4	57.1	9	60	13	59.1	X^2 =0.016
>10%	3	42.9	6	40	9	40.9	P value =0.899

Out of 22 cases of muscle tissue included biopsies, 7 cases were diagnosed as stage of pT_a/T_1 and 15 cases were stage of T_2 and above.

Among 7 cases, 4 cases (57.1%) shows decreased (<10%) of expression of p63 and 3 cases (42.9%) shows increased (>10%) expression of p63.

Among 15 cases of stage T_2 and above, 9 cases (60%) shows decreased expression of p63 and 6 cases (40%) shows increased expression of p63.

Predominantly both stages of tumour shows decreased (< 10%) expression of p63 in tumour cells. p63 expression did not depend on the stage of the tumour.

This study does not show statistically significant correlation between p63 expression and stage of the tumour. P value was 0.899.

Table: 13: Correlation between the expression of Ki-67 and p63 expression

p63 expression	**Ki-67 expression**				**Total**		**Statistical Inference**
	<20% expression		>20% expression				
	n	%	n	%	n	%	
<10%	2	10	20	66.7	22	44	X^2 =15.639 P value =0.000
>10%	18	90	10	33.3	28	56	

Among 20 cases of decreased (<20%) expression of Ki- 67, 2 cases (10%) shows decreased (<10%) expression of p63, remaining 18 cases (90%) shows increased (>10%) expression of p63.

Out of 30 cases of increased (>20%) expression of Ki -67, 20 cases (66.7%) shows decreased (<10%) expression of p63 and 10 cases (33.3%) shows increased (>10%) expression of p63.

The expression of Ki-67 was inversely proportionate to p63 expression. This observation shows p value of 0.000 which was statistically significant.

DISCUSSION

DISCUSSION

In this study, we evaluated the IHC expression of Ki-67 and p63 in urothelial carcinoma and also looked for correlation of their expression with various clinico pathological variables like age, gender, grade and stage of the tumour.

This study evaluated the 50 formalin fixed paraffin embedded tissue blocks of urothelial carcinoma for expression of Ki-67 and p63 in tumour cells.

Among 50 cases of UC, majority of the patients were males 62% (n =31 cases) and above 60 years 58 % (n = 29 cases). A similar observation regarding gender wise distribution of UC found in study done by Thakur et al [(101)] and age wise distribution was found in the other studies done by Gupta et al [(103)] and Biswas RR et al. [(104)]

Out of 50 cases, males were 62% (n = 31 cases) and females were 38% (n = 19 cases) accounting to male to female ratio 1.6: 1.The mean age at diagnosis was 62 years. These observations were similar with Mungan et al [(105)] and Quintero et al [(106)].

Among 50 cases, low grade urothelial carcinoma were 38% (n =19 cases) and high grade urothelial carcinoma were 62% (n =31 cases).Majority of the low grade and high grade urothelial carcinoma were in the age group of above 60 years forming 57.9% (n= 11 cases), 58.1% (n= 18 cases) respectively. This observed result was not similar with Joshi et al. [(107)]

Out of 50 UC cases, 94% were TURBT specimens (n =47 cases) and 6% were cystectomy (n = 3 cases). Invasive nature of the tumour was assessed in muscle tissue included biopsy specimens.

Out of 22 cases of muscle tissue included biopsies, low grade urothelial carcinoma were 18% (n =4 cases) and high grade urothelial carcinoma were 82% (n =18 cases). Among 4 low grade urothelial carcinoma cases, 1 case (25%) show muscle invasion and 3 cases (75%) were non muscle invasive. Out of 18 cases of high grade urothelial carcinoma 14 cases (78%) shows muscle invasion, remaining 4 cases (22%) were non muscle invasive.

Variable frequency of muscle invasive and non muscle invasive urothelial carcinoma was noted in several studies. This study shows frequency of non muscle invasive carcinoma was 32% (n =7 cases) and muscle invasive carcinoma was 68% (n =15 cases). This observation was similar with Gupta et al [103] which was conducted on 561 bladder carcinoma specimens 26% were non muscle invasive urothelial carcinoma and not similar with Thakur et al [101] which was conducted on 110 cases of bladder carcinoma 74% shows non muscle invasive tumour .

The incidence of high grade and muscle invasive urothelial carcinoma was higher compared with low grade, non muscle invasive urothelial carcinoma. It may due to delayed medical attention. Among the low grade urothelial carcinoma most of them are non muscle invasive where as high grade urothelial carcinoma most frequently shows muscle invasion.

Out of 22 cases, 10 cases (45%) were in the age group of 40 -60 years, 12 cases (55%) were in above 60 years. Invasive nature of tumour was more in high grade tumour and amounting to (out of 18 cases of high grade urothelial carcinoma 14 cases show muscle invasion) than low grade 25% (out of 4 cases low grade urothelial

carcinoma 1 case shows muscle invasion).Most of the high grade urothelial carcinoma were diagnosed above the age of 60 years amounting for 61% (out of 18 cases, 11 cases were above 60 years).

Out of 22 cases 7 cases were in pT_a / T_1 (4 cases (57%) were in the age group of 40 – 60 years, 3 cases (43%) were in above 60 years) and 15 cases were $_pT_2$ and above in stage (6 cases (40%) were in the age group of 40 – 60 years, 9 cases (60%) were in above 60 years).

The tumour grade directly correlates with advanced age and invasiveness of the tumour was directly proportionate with grade and increasing age. This observation was found comparable with Yuvaraja TB et al study. [108]

Out of 50 cases, decreased proliferation of Ki-67 noted in 20 cases whereas increased proliferation noted in 30 cases. Majority of the increased proliferation 60 %, (n =18 cases) and decreased proliferation 55% (n =11 cases) were seen in above 60 years. Out of 20 cases of decreased proliferation and 30 cases of increased proliferation of Ki-67, majority of the cases were males and accounting for 70% (n =14 cases), 56.7% (n =17cases) respectively. This study shows no statistically significant correlation between Ki- 67 expression with age and gender. Similar observation was seen in Lujia Wang et al [109] and Krishna kanth et al. [110]

In this study included 50 cases, 80% of low grade urothelial carcinoma (n =16 cases) were shows decreased proliferation of Ki -67 and 90% of the high grade urothelial carcinoma (n =31 cases) shows increased proliferation of Ki- 67 index. This observation shows significant correlation of Ki-67 expression with histological grade

of the tumour and this result was similar with Lujia Wang et al [109] and Vitaly Margulis et al. [111]

Out of 22 cases of muscle tissue included biopsies, 15 cases shows muscle invasion and 7 cases did not show muscle invasion. 86.7% (n = 13 cases) of muscle invasive urothelial carcinoma and 57.1% (n = 4 cases) of non muscle invasive urothelial carcinoma shows increased proliferation of Ki-67. So Ki-67 expression did not show significant correlation with invasive nature of urothelial carcinoma. This result was different from Lujia Wang et al. [109]

In this study out of 50 cases, 22 cases showed decreased expression of p63 and 28 cases showed increased expression. In 22 cases of decreased expression of p63 males 14 cases (63.6%) and in 28 cases of increased expression of p63 , males were 60.7% (17 cases).These observation does not show statistical significant correlation between the expression of p63 with age and gender of the patient. Similar results were observed by Afaf T. Elnashar et al. [112]

Out of 50 cases, most of the low grade urothelial carcinoma (64.3%, n=19 cases) shows increased expression of p63 whereas most of the high grade urothelial carcinoma (95.5%, n =21 cases) shows decreased expression. This result was statistically significant and in concordance with other study done by Afaf T. Elnashar et al [112] and Sayed Abdel Raheem. [113]

Among 22 cases of muscle tissue included biopsies, 15 cases shows muscle invasion and 7 cases does not shows muscle invasion. Out of 15 cases of muscle invasive urothelial carcinoma, 60% (n =9 cases) shows decreased expression. Out of 7

cases of non muscle invasive urothelial carcinoma 57.1% (n = 4 cases) cases shows decreased expression of p63. This observation shows expression of p63 does not correlate with invasive nature of urothelial carcinoma and also statistically non significant. This result was similar with Ali koyuncuer. [114]

Out of 20 cases of decreased proliferation of Ki -67, 18 cases (90%) shows increased expression of p63 and 30 cases of increased proliferation of Ki-67, 20 cases (n = 66.7) shows decreased expression of p63. This result shows statistically significant inverse correlation between Ki -67 and p63 expression.

CONCLUSION

CONCLUSION

The present study analysed the Ki-67 and p63 expression in varying grades of urothelial carcinoma. The following conclusions were arrived at.

The incidence of urothelial carcinoma was most common above 60 years of age and males were affected more commonly. As age advances, the histological grade and invasive nature of the tumour were higher.

Even though urothelial carcinoma was less common in females, they usually presented with higher grade and stage.

Low grade urothelial carcinoma shows lower proliferation (<20%) of Ki-67 and increased expression (>10%) of p63, where as high grade urothelilal carcinoma shows higher proliferation (>20%) of Ki-67 and decreased expression (<10%) of p63 in tumour cells.

We were able to demonstrate the statistically significant inverse correlation between the Ki-67 and p63 expression in low grade and high grade urothelial carcinoma.

Expression of ki-67 and p63 occur independently of age, gender and muscle invasiveness of the tumour.

These results are in parallel with other published studies.

The increased proliferation of Ki-67 and decreased expression of p63 in low grade urothelial carcinoma cases may suggest aggressive clinical course and these cases may have increased chance of tumour recurrence and progression, so need further appropriate adjuvant treatment.

This study shows statistical association between the expression of Ki-67 and p63 in varying grades of urothelial carcinoma and some has clinical significance. Survival rate cannot be assessed because this study lacks follow up data.

More studies with large sample size involving diverse population groups may be necessary to look for association.

We hope to address these limitations in future studies.

Colour plates

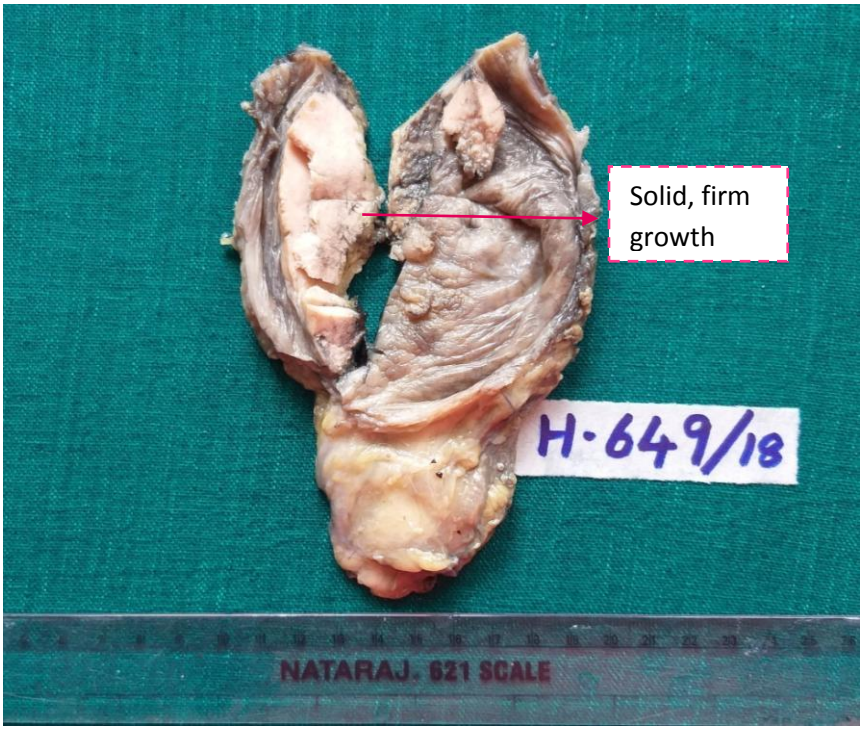


Figure: 8Cystectomy - Invasive urothelial carcinoma

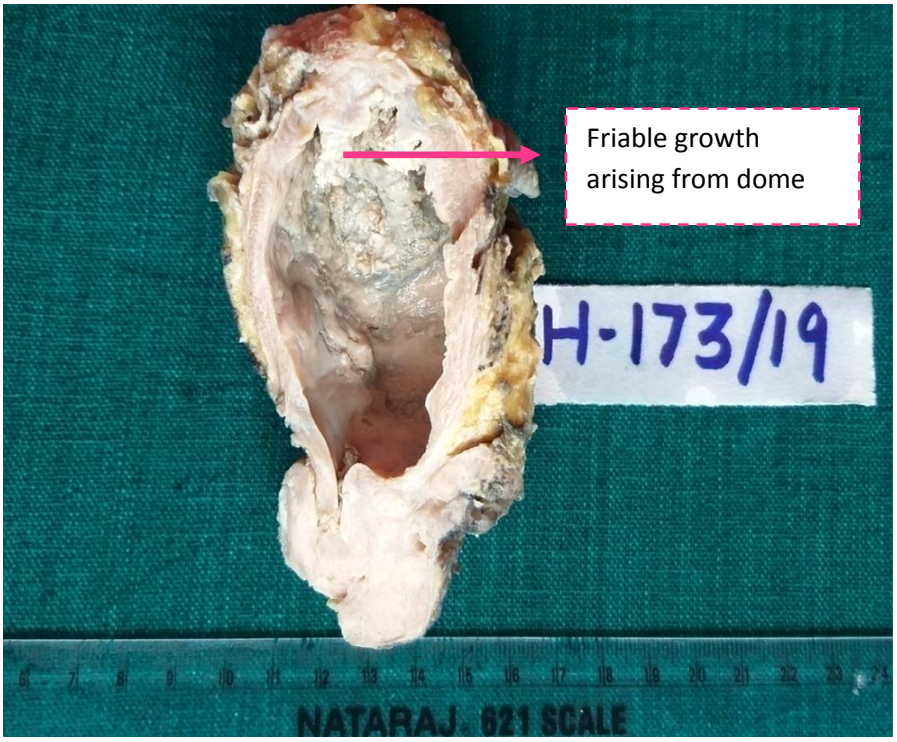


Figure:9 Cystectomy - Invasive urothelial carcinoma

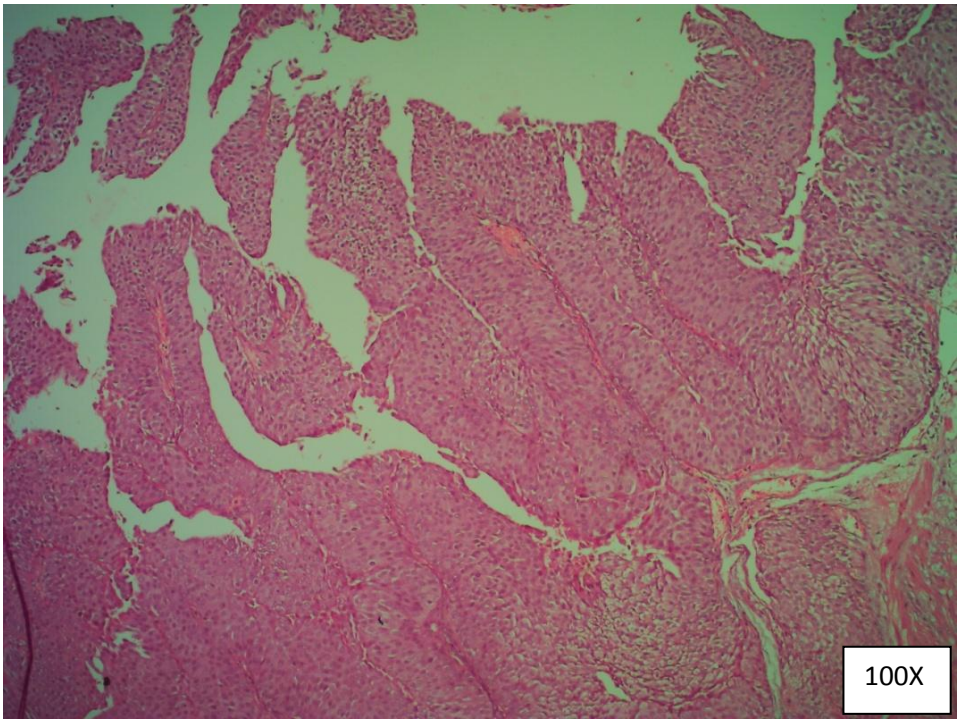


Figure: 10 Low grade urothelial carcinoma H&E (H-269/19)

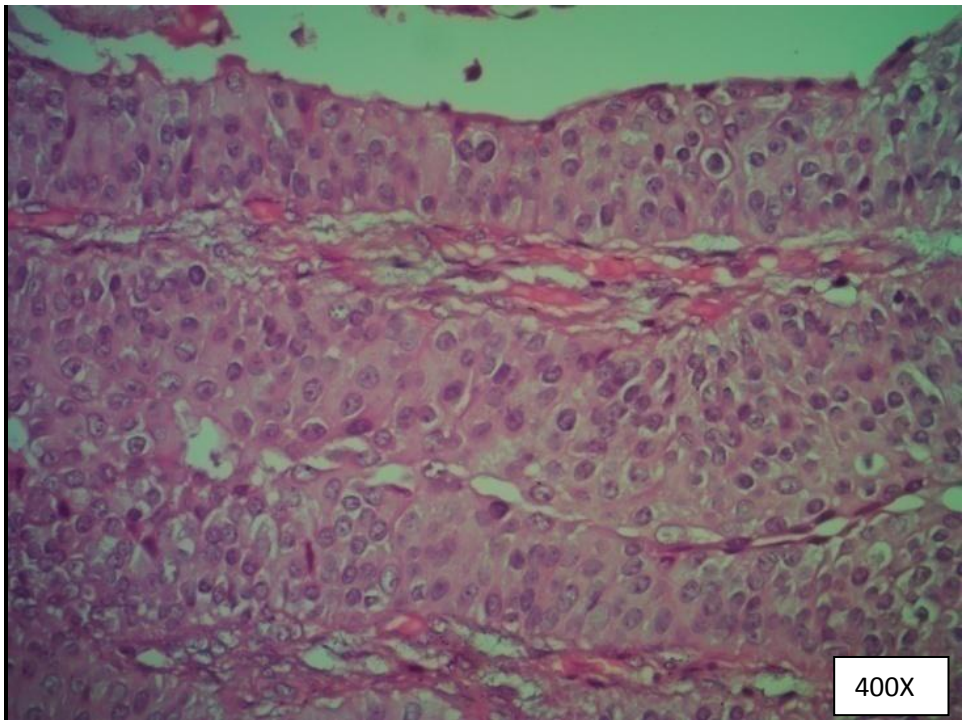


Figure: 11 Low grade urothelial carcinoma H&E (H-269/19)

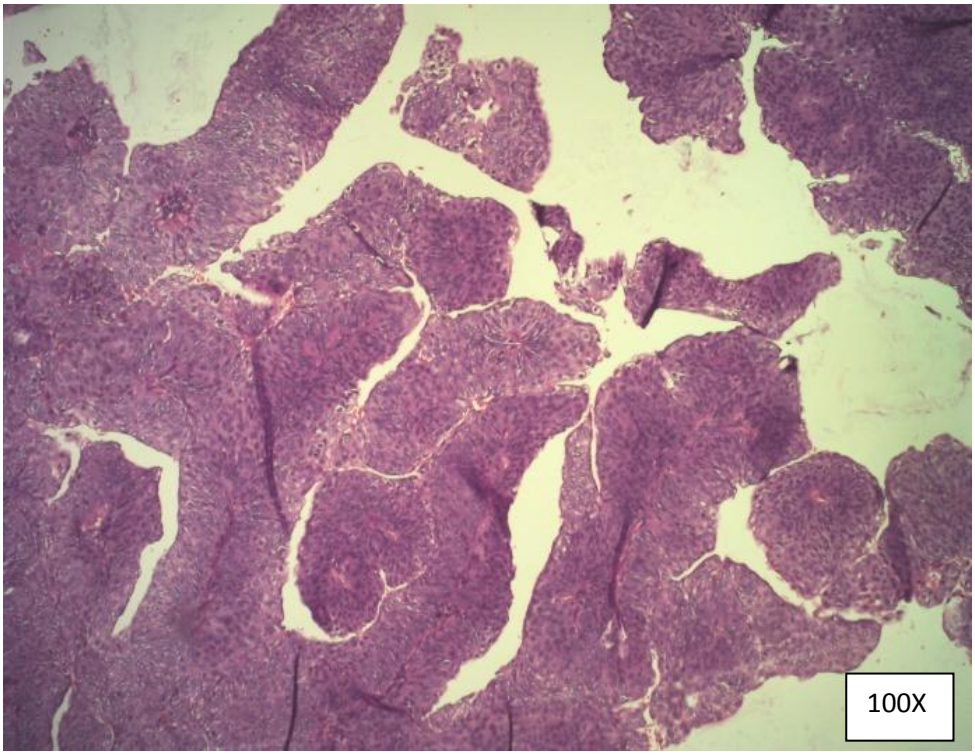


Figure: 12 Low grade urothelial carcinoma H&E (H-5133/16)

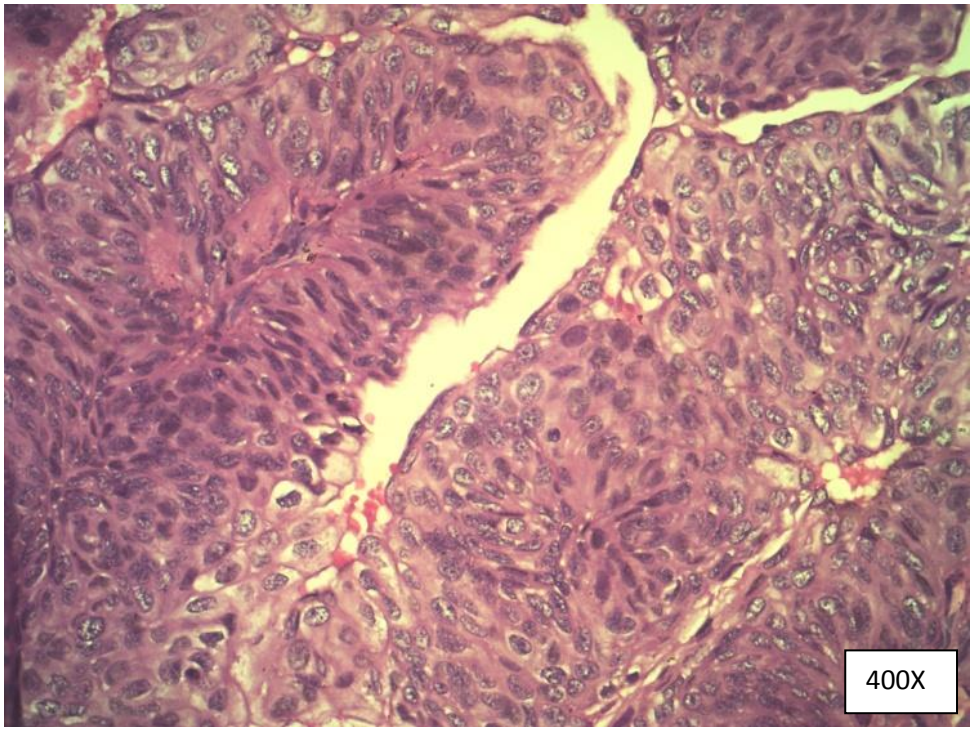


Figure: 13 Low grade urothelial carcinoma H&E (H-5133/16)

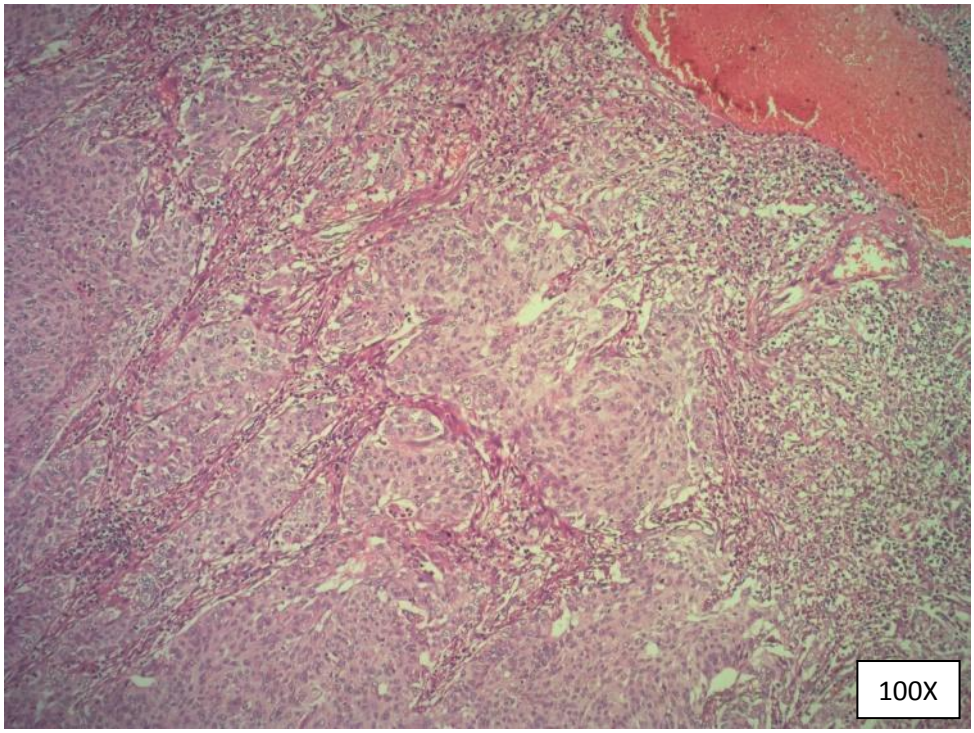


Figure: 14 Low grade urothelial carcinoma - Lamina propria invasion H& E (H-1215/17)

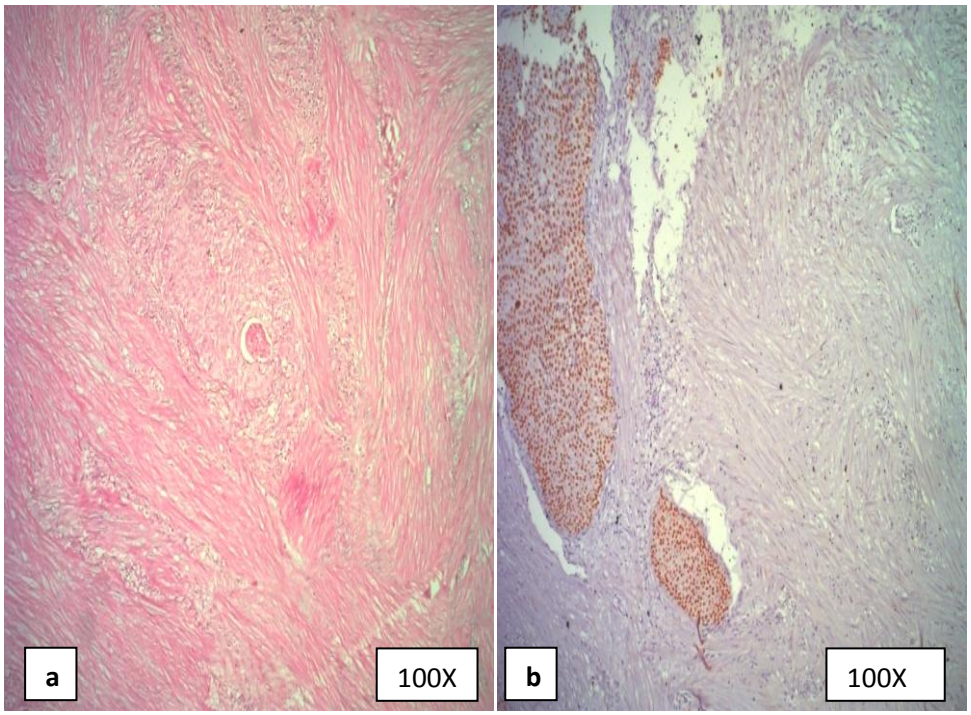


Figure: 15 Low grade urothelil carcinoma - muscle invasion (H -649/19) a) H&E b) IHC expression of p63 in tumour cells

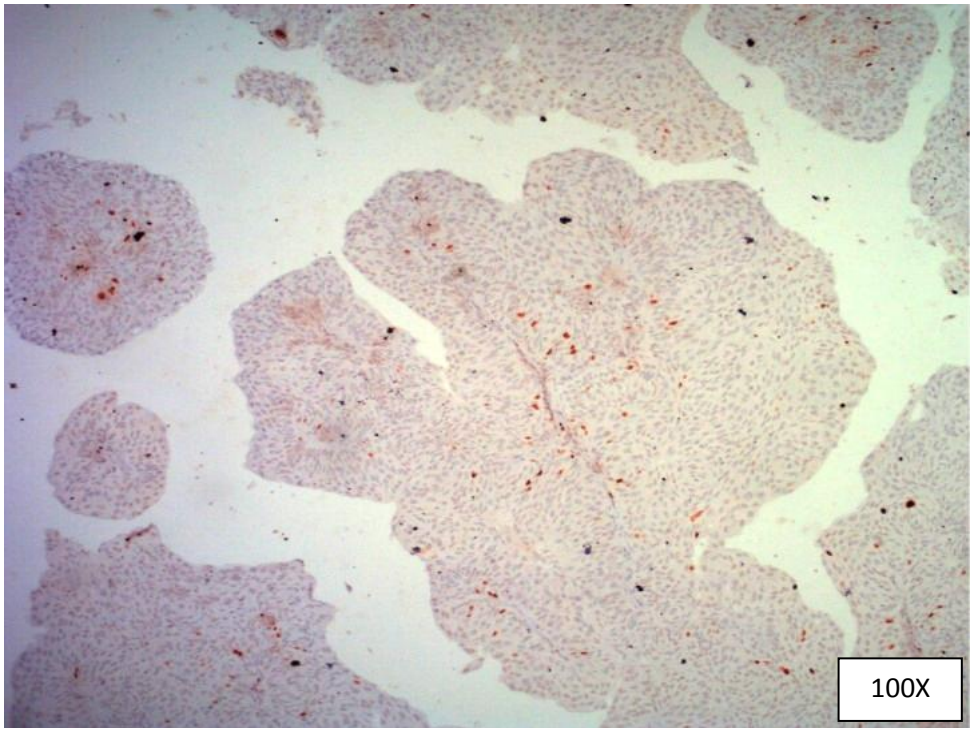


Figure: 16 Lower expression (<20%) of Ki -67 in low grade urothelial carcinoma (H – 2593/17)

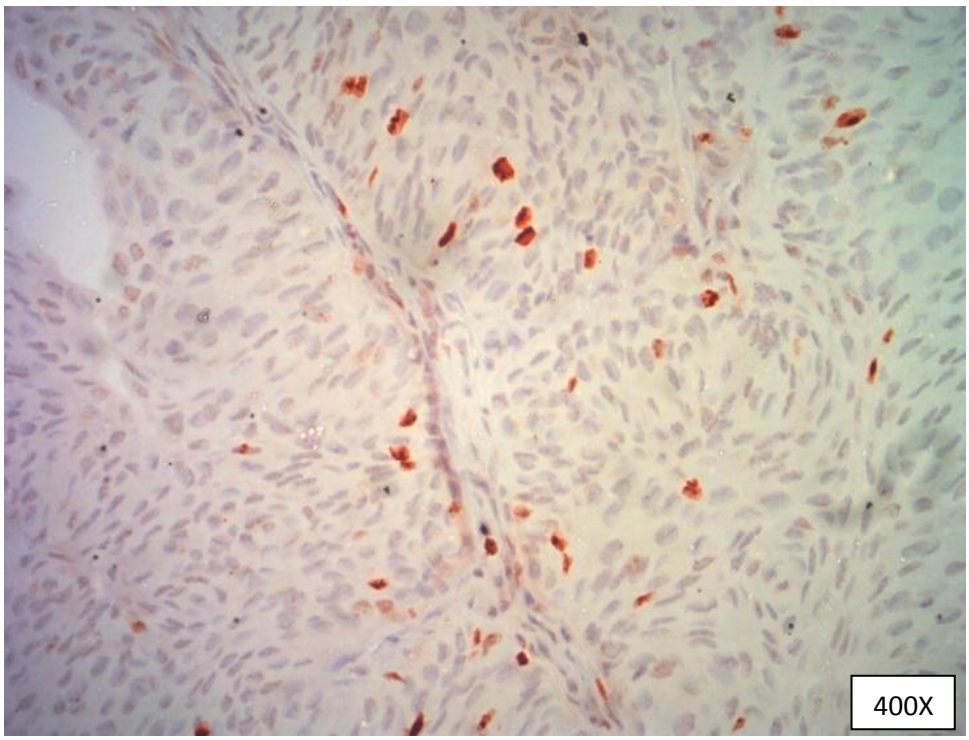


Figure: 17 Lower expression (<20%) of Ki -67 in low grade urothelial carcinoma (H – 2593/17)

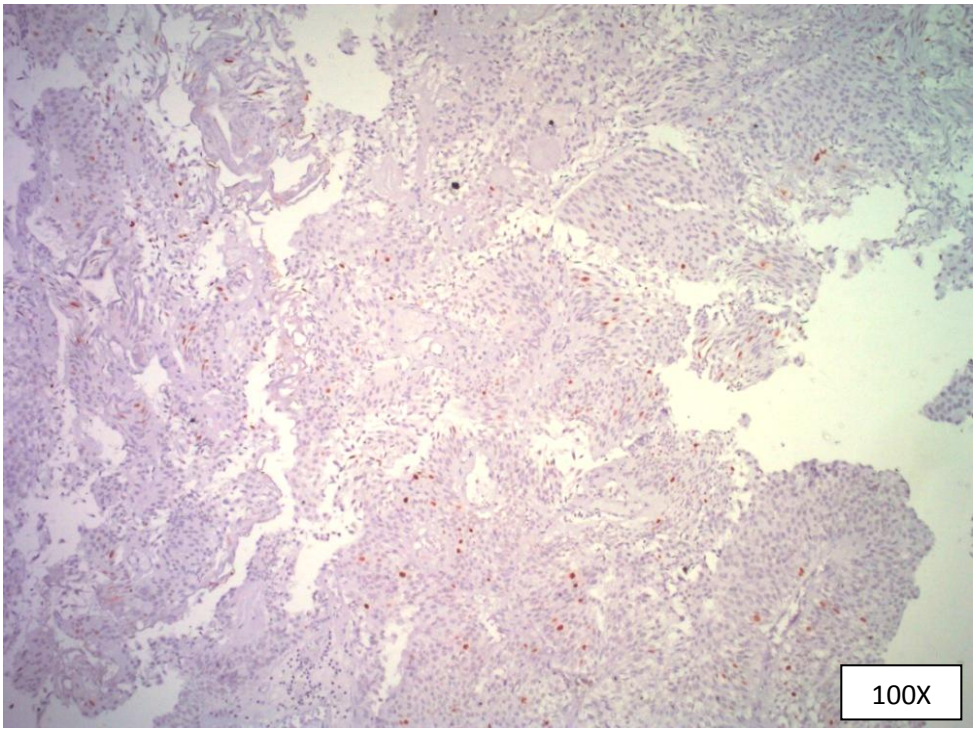


Figure: 18 Lower expression (<20%) of Ki -67 in low grade urothelial carcinoma (H – 1490/19)

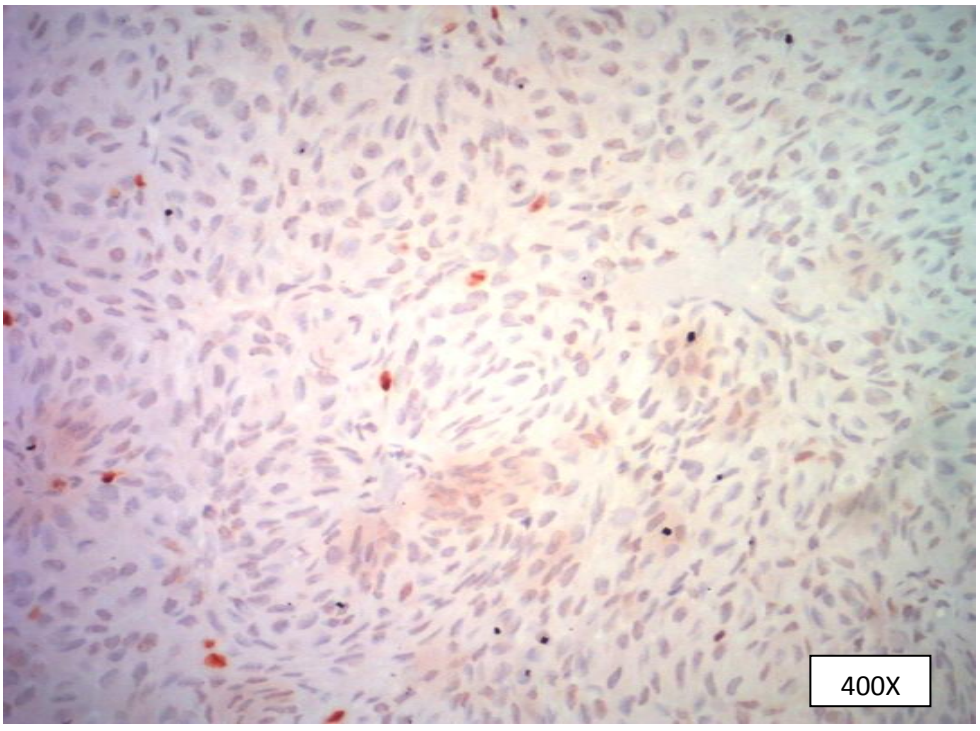


Figure: 19 Lower expression (<20%) of Ki -67 in low grade urothelial carcinoma (H – 1490/19)

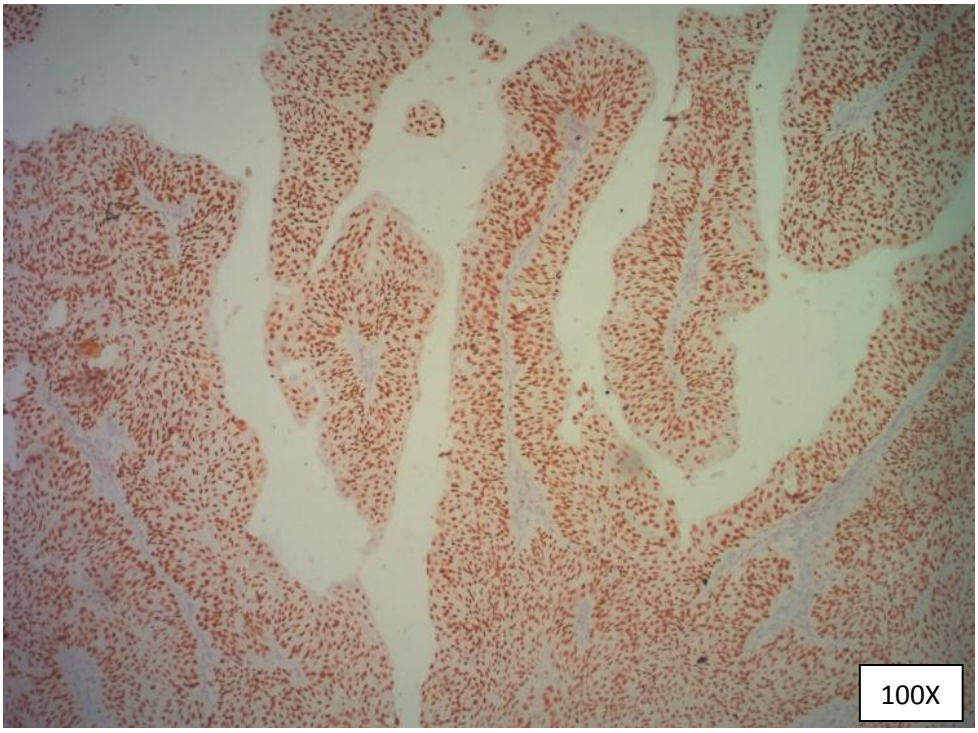


Figure: 20 Increased expression (>10%) of p63 in low grade urothelial carcinoma (H – 4089/17)

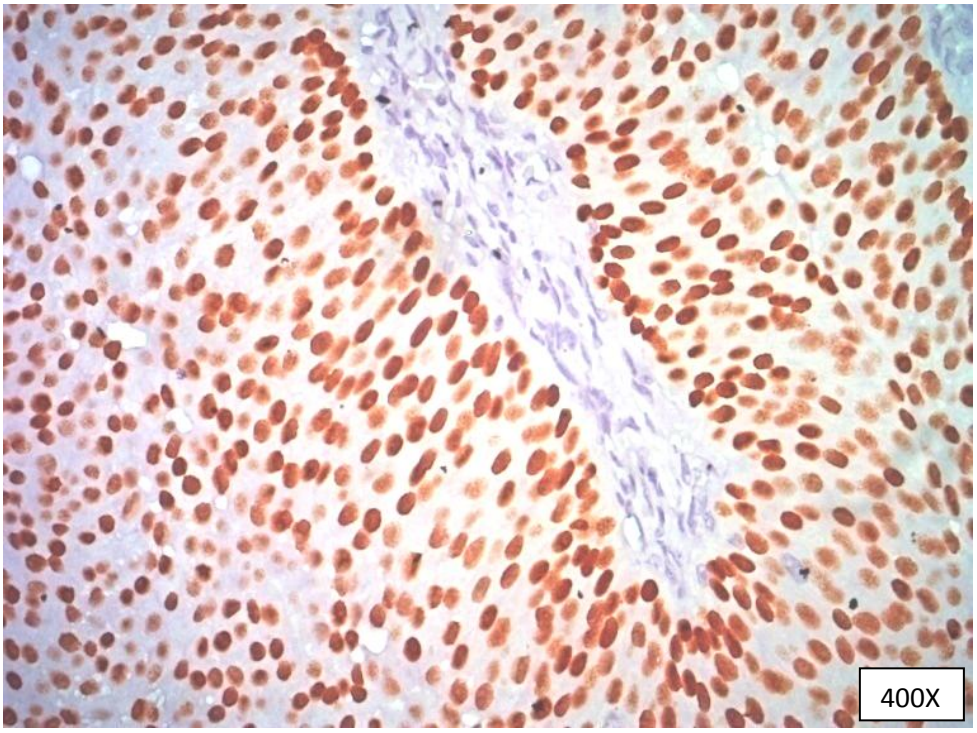


Figure: 21 Increased expression (>10%) of p63 in low grade urothelial carcinoma (H – 4089/17)

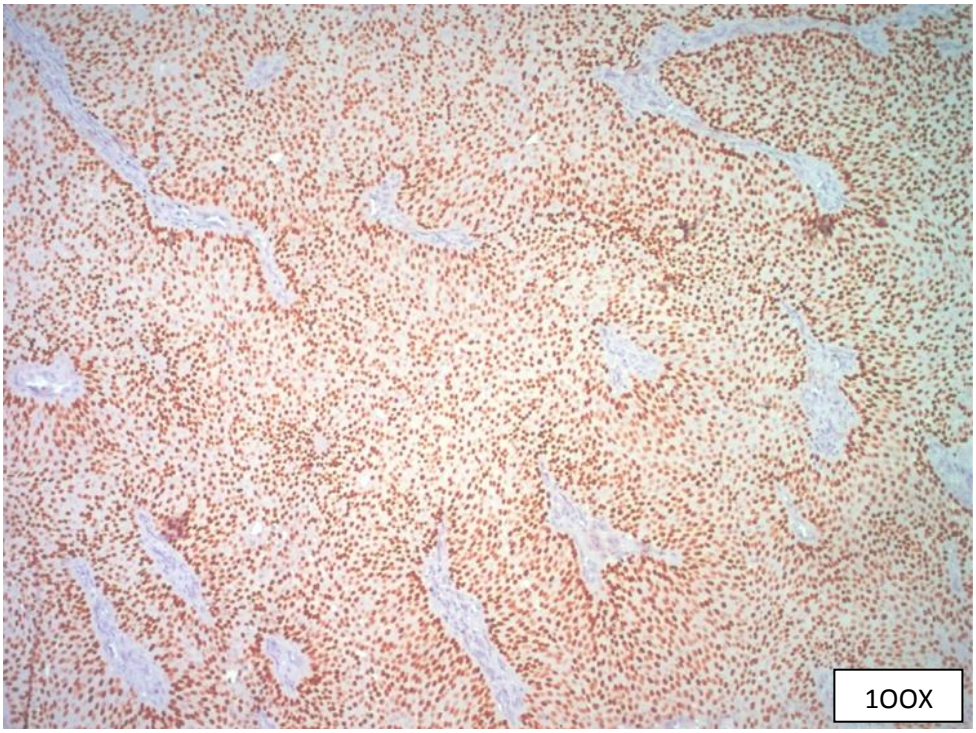


Figure: 22 Increased expression (>10%) of p63 in low grade urothelial carcinoma (H – 5589/18)

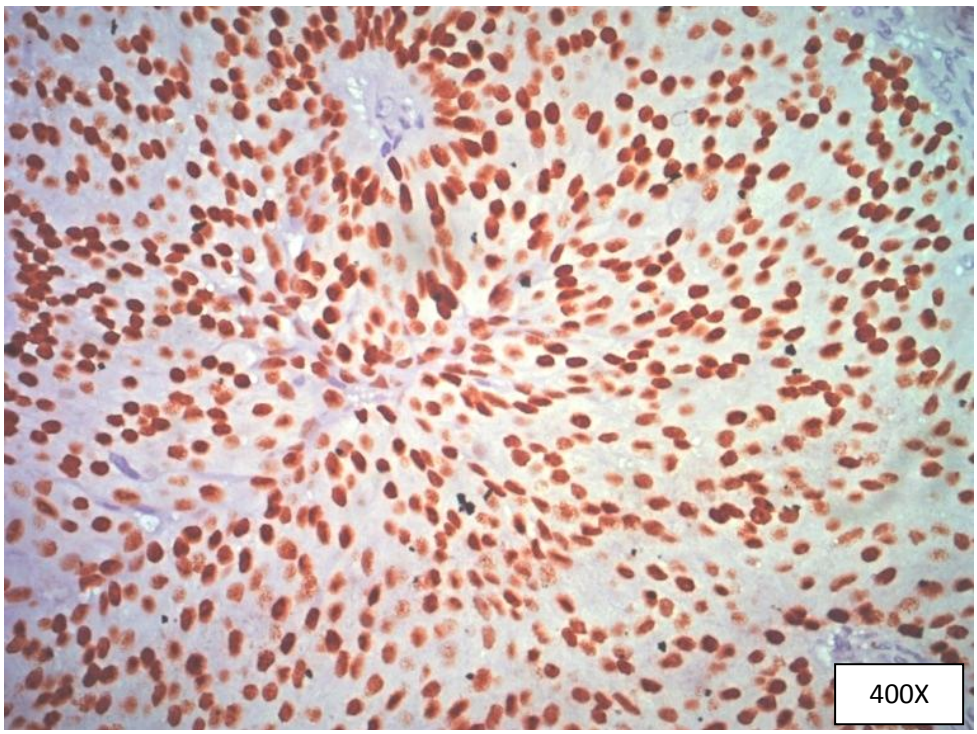


Figure: 23 Increased expression (>10%) of p63 in low grade urothelial carcinoma (H – 5589/18)

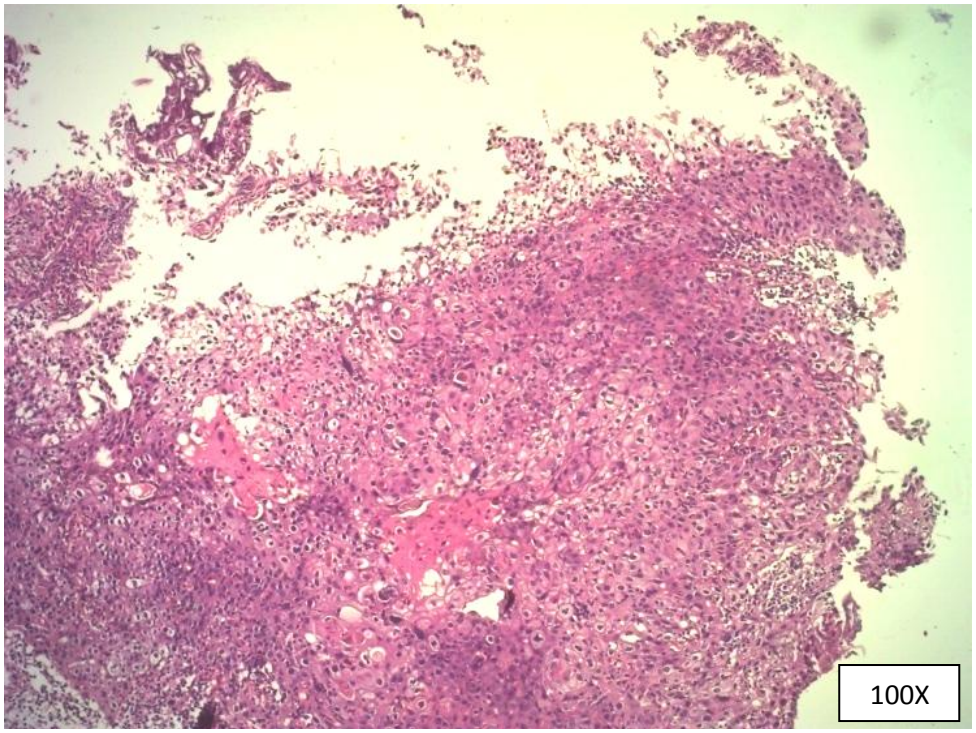


Figure: 24 High grade urothelial carcinoma H&E (H-1578/17)

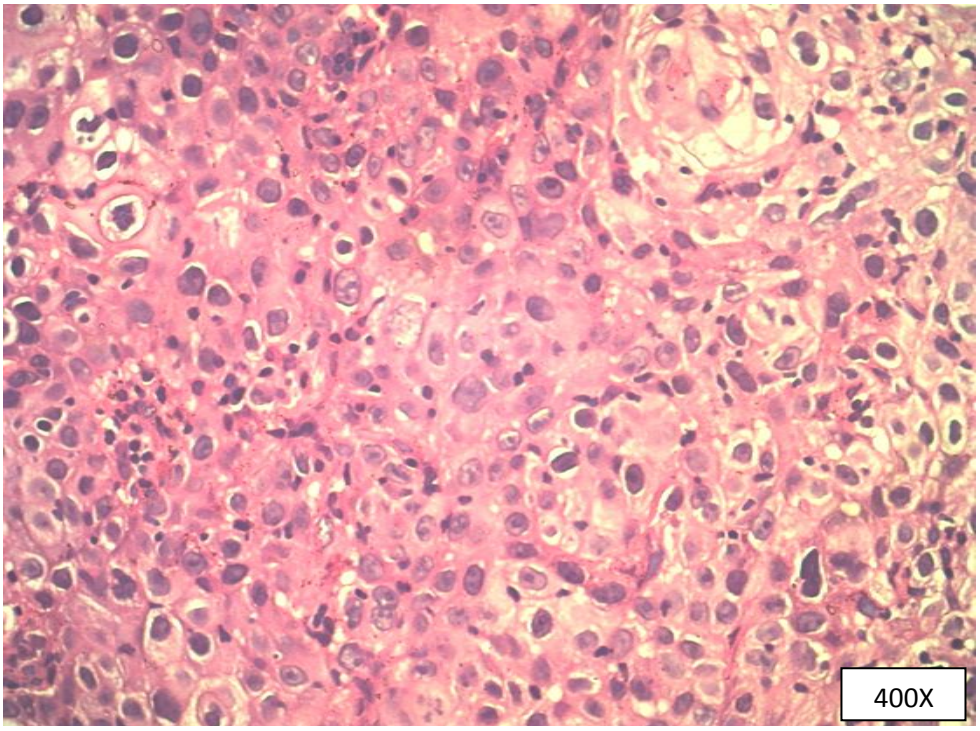


Figure: 25 High grade urothelial carcinoma H&E (H-1578/17)

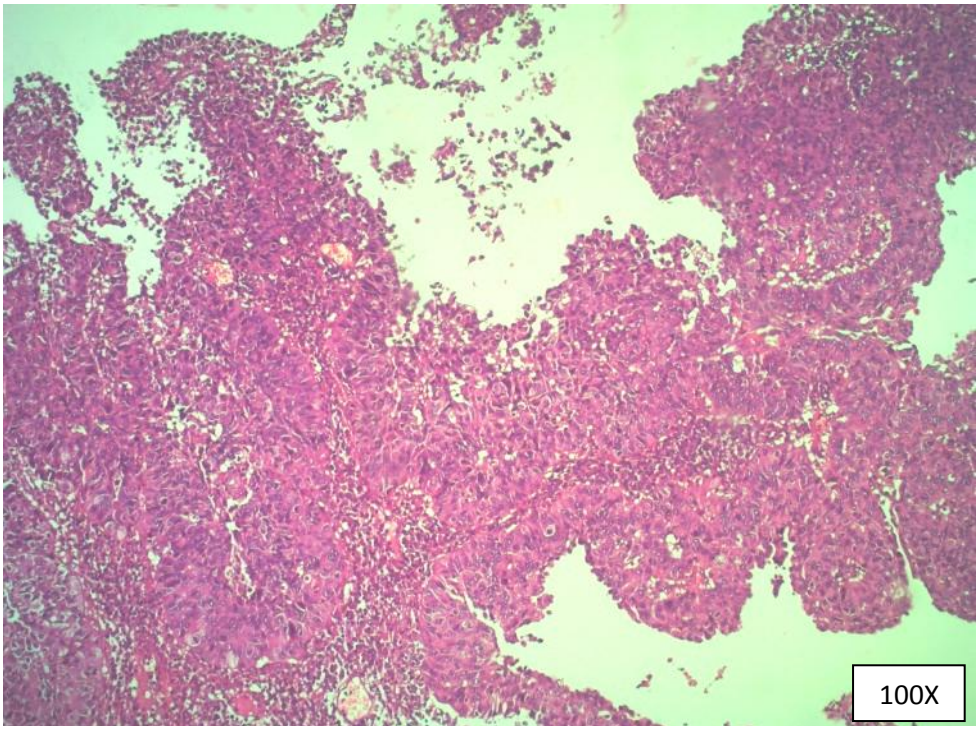


Figure: 26 High grade urothelial carcinoma H&E (H-549/19)

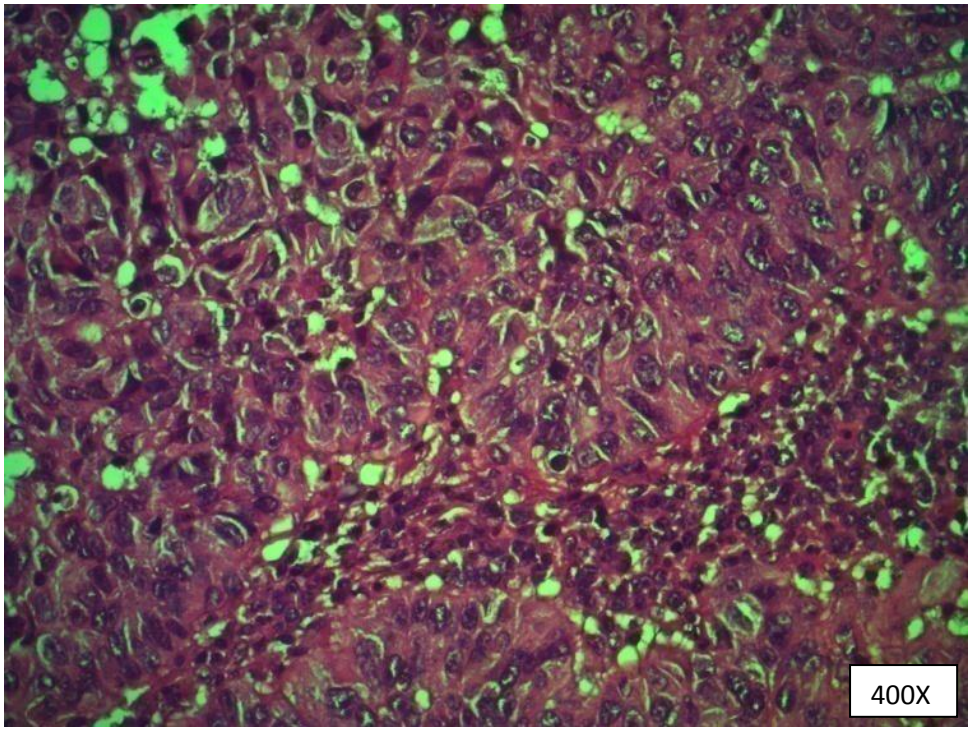


Figure: 27 High grade urothelial carcinoma H&E (H-549/19)

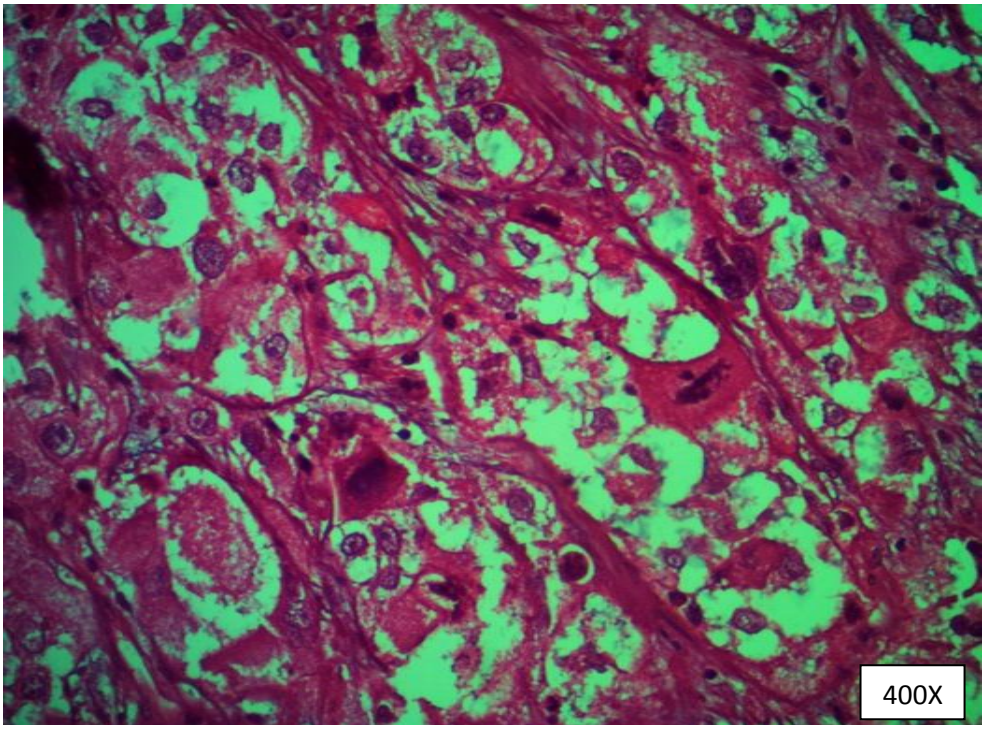


Figure: 28 High grade urothelial carcinoma – prominent mitosis H&E (H-549/19)

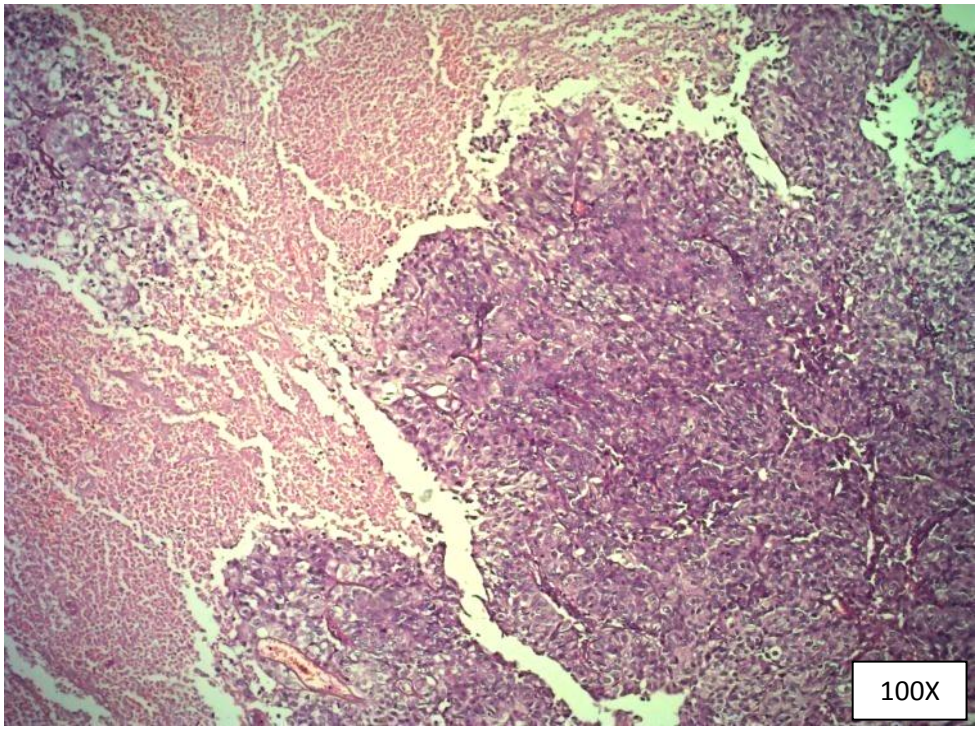


Figure: 29 High grade urothelial carcinoma – necrosis H&E (H- 1085/19)

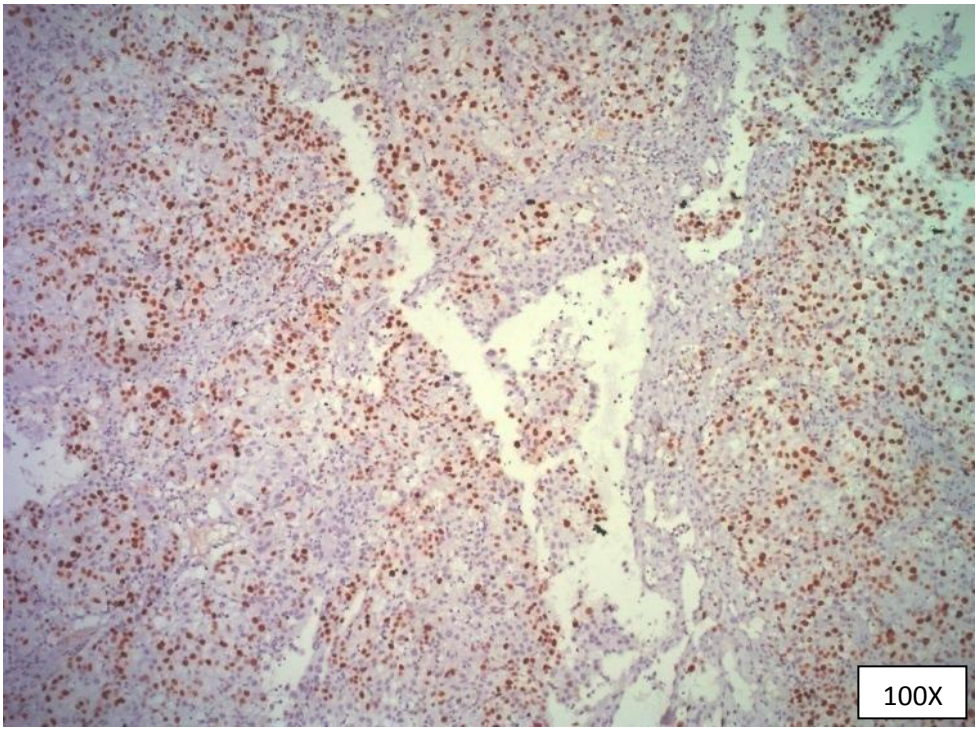


Figure: 30 High grade urothelial carcinoma – increased (>20%) expression of Ki -67 (H – 3854/16)

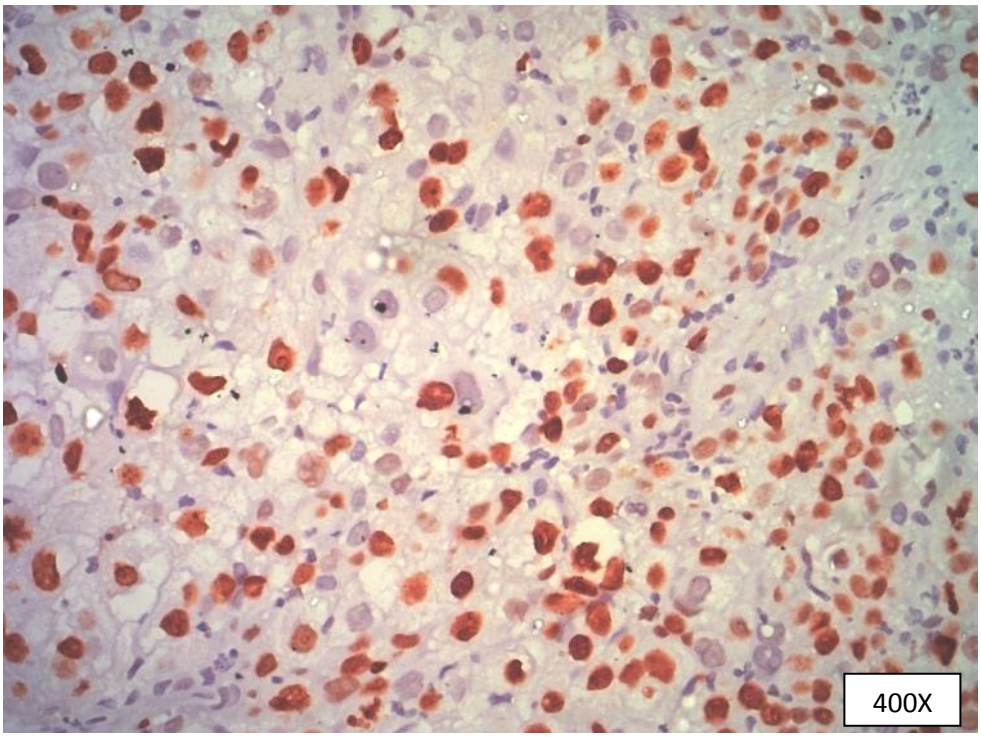


Figure: 31 High grade urothelial carcinoma – increased (>20%) expression of Ki -67 (H – 3854/16)

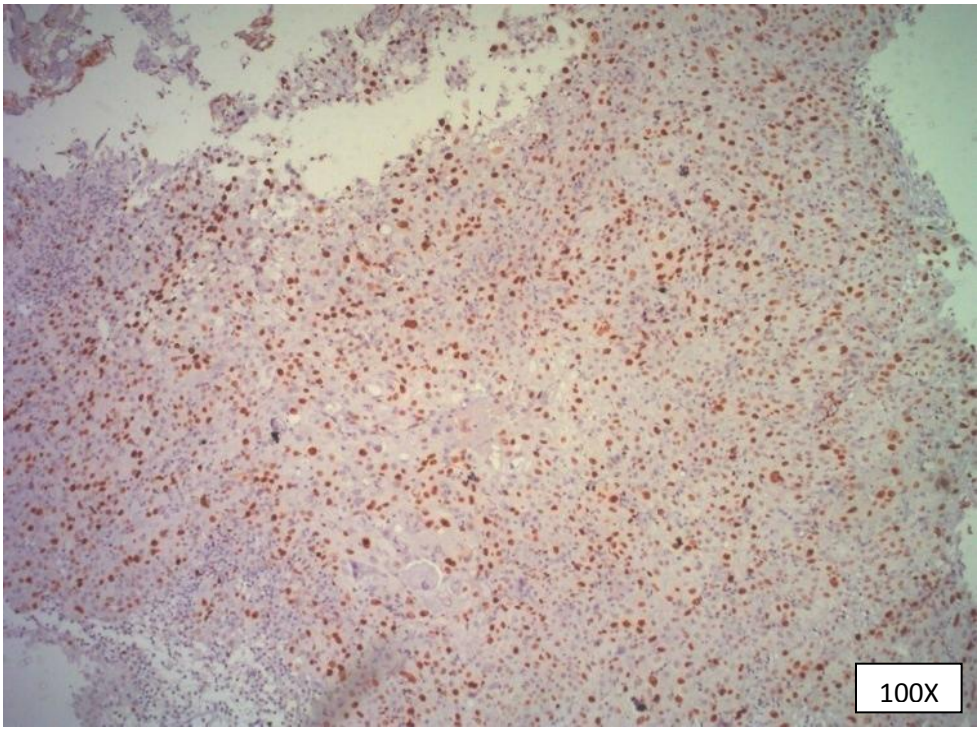


Figure: 32 High grade urothelial carcinoma – increased (>20%) expression of Ki -67 (H – 5198/19)

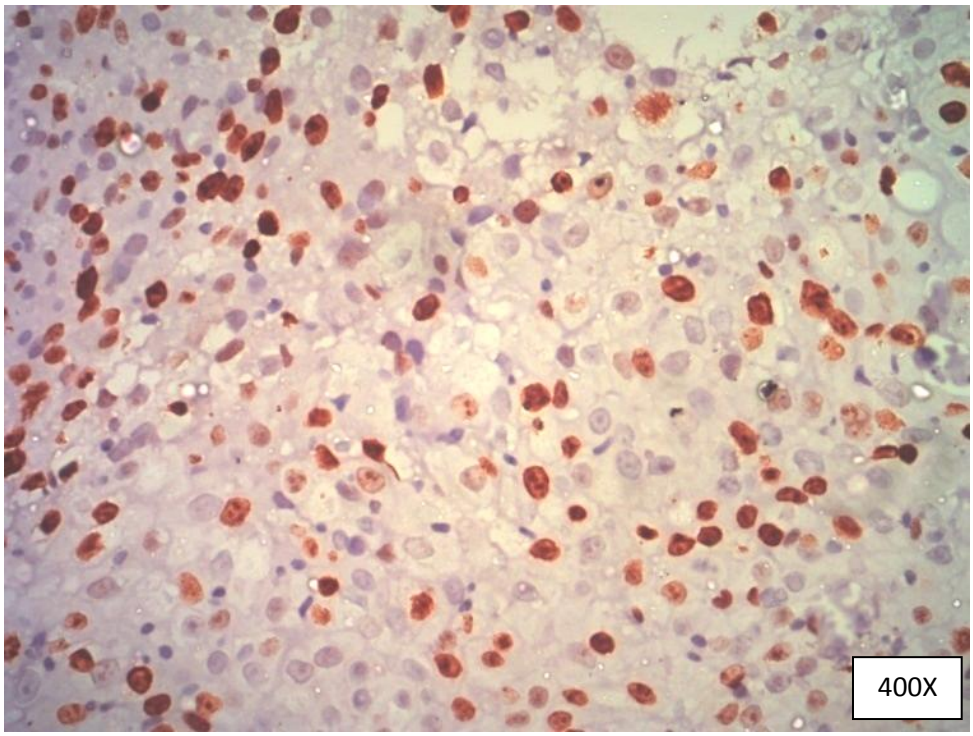


Figure: 33 High grade urothelial carcinoma – increased (>20%) expression of Ki -67 (H – 5198/19)

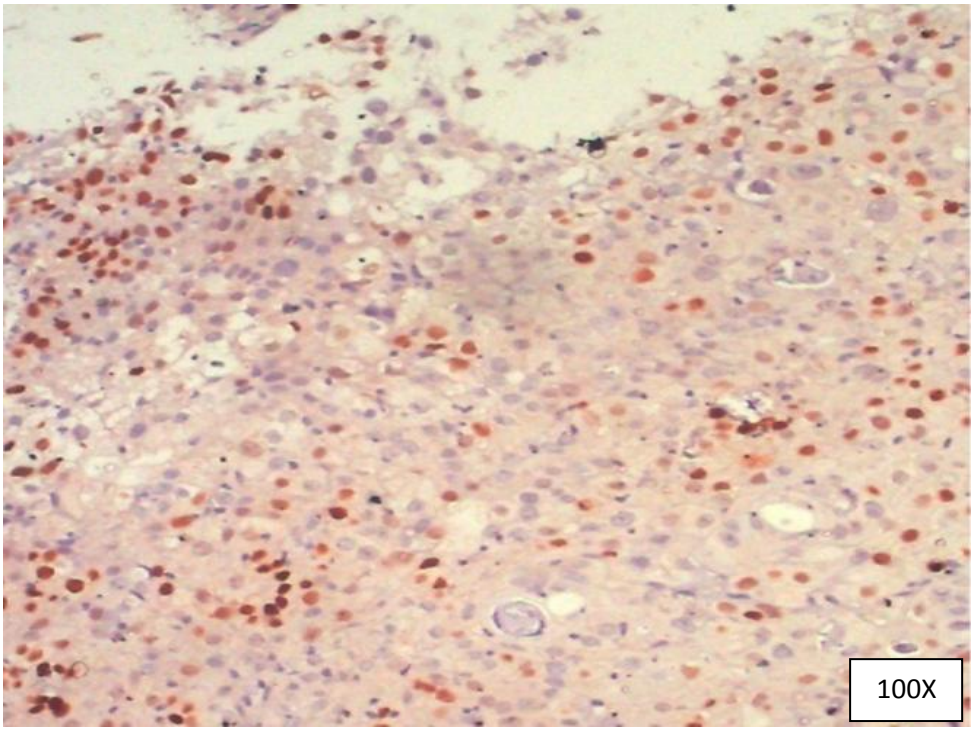


Figure: 34 High grade urothelial carcinoma –decreased (< 10%) expression of p63 (H – 5049/17)

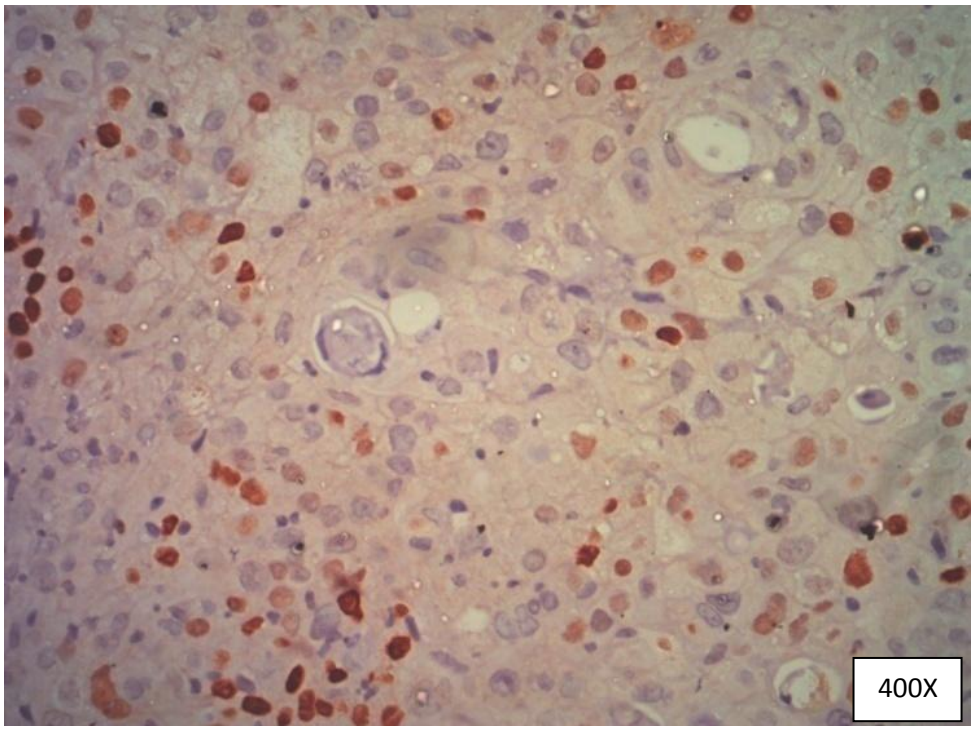


Figure: 35 High grade urothelial carcinoma –decreased (< 10%) expression of p63 (H – 5049/17)

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ANNEXURE

I. MASTER CHART

ANNEXURE: I

MASTER CHART

SL.NO	HPE NO	AGE -YEARS	SEX	PROCEDURE	MUSCLE TISSUE IN BIOPSY	MUSCLE INVASION	HISTOPATHOLOGICAL GRADE	Ki 67	P63
1.	H-63/15	55	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
2.	H- 3131/15	60	FEMALE	TURBT	PRESENT	ABSENT	HIGH	>20%	<10%
3.	H- 3055/15	46	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
4.	H-378/15	59	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
5.	H-1885/15	56	MALE	TURBT	ABSENT	ABSENT	HIGH	<20%	>10%
6.	H- 1873/15	47	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
7.	H-3900/15	70	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
8.	H-2959/15	65	FEMALE	TURBT	ABSENT	ABSENT	HIGH	>20%	>10%
9.	H-3684/15	70	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
10.	H-5133/16	80	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
11.	H-2323/16	64	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
12.	H-3512/16	81	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
13.	H-4484/16	60	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	>10%
14.	H-3854/16	68	FEMALE	CYSTECTOMY	PRESENT	PRESENT	HIGH	>20%	<10%
15.	H-6030/17	50	FEMALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
16.	H- 4774/17	63	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	>10%
17.	H- 5049/17	65	MALE	TURBT	PRESENT	ABSENT	HIGH	>20%	<10%
18.	H-5033/17	66	MALE	TURBT	PRESENT	ABSENT	HIGH	>20%	<10%
19.	H-4523/17	75	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
20.	H-2593/17	55	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
21.	H-4880/17	69	MALE	TURBT	ABSENT	ABSENT	HIGH	<20%	>10%
22.	H- 4089/17	50	MALE	TURBT	PRESENT	ABSENT	LOW	<20%	>10%
23.	H-3347/17	59	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
24.	H-1215/17	70	FEMALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
25.	H- 144/17	65	FEMALE	TURBT	ABSENT	ABSENT	LOW	>20%	>10%
26.	H-129/17	50	MALE	TURBT	PRESENT	ABSENT	HIGH	>20%	<10%
27.	H-4300/17	75	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%

28.	H-698/17	68	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	>10%
29.	H-4666/17	65	FEMALE	TURBT	PRESENT	PRESENT	HIGH	<20%	>10%
30.	H-1578/17	35	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	>10%
31.	H-3159/17	60	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
32.	H-4067/17	63	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
33.	H-5198/18	60	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
34.	H-5589/18	27	FEMALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
35.	H-6078/18	80	FEMALE	TURBT	ABSENT	ABSENT	HIGH	>20%	>10%
36.	H-4053/18	50	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
37.	H- 2783/18	75	MALE	TURBT	PRESENT	ABSENT	LOW	<20%	>10%
38.	H-1952/18	70	FEMALE	TURBT	PRESENT	PRESENT	HIGH	>20%	<10%
39.	H-1325/18	56	FEMALE	TURBT	ABSENT	ABSENT	HIGH	>20%	<10%
40.	H- 649/18	52	FEMALE	CYSTECTOMY	PRESENT	PRESENT	LOW	<20%	>10%
41.	H-549/19	79	MALE	TURBT	PRESENT	PRESENT	HIGH	>20%	>10%
42.	H- 269/19	74	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	>10%
43.	H- 173/19	49	FEMALE	TURBT	ABSENT	ABSENT	HIGH	>20%	>10%
44.	H-1085/19	75	MALE	TURBT	ABSENT	ABSENT	HIGH	>20%	>10%
45.	H-1226/19	70	MALE	TURBT	ABSENT	ABSENT	LOW	>20%	>10%
46.	H-1490/19	48	FEMALE	TURBT	PRESENT	ABSENT	LOW	<20%	>10%
47.	H-3207/19	70	MALE	TURBT	ABSENT	ABSENT	LOW	>20%	>10%
48.	H-916/19	76	MALE	CYSTECTOMY	PRESENT	ABSENT	HIGH	<20%	<10%
49.	H-1004/19	66	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	<10%
50.	H-2446/19	84	MALE	TURBT	ABSENT	ABSENT	LOW	<20%	<10%

II. IHC STAINING PROCEDURE

ANNEXURE: II

IHC STAINING PROCEDURE

Tissue Preparation (Formalin Fixed, Paraffin-embedded Sections):

1. Cut sections at 4um and place on pre-cleaned and positively charged microscope slides.
2. Heat in a tissue-drying oven for 45 minutes at 60°C.

Deparaffinization and Rehydration:

3. Wash slides 2 times in Xylene for 3 minutes each time at room temperature (RT).
4. Wash slides in Xylene 1:1 with 100% ethanol for 3 minutes at RT.
5. Wash slides 2 times in 100% ethanol for 3 minutes each at RT.
6. Wash slides 2 times in 95% ethanol for 3 minutes each at RT.
7. Wash slides in 70% ethanol for 3 minutes at RT.
8. Wash slides in 50% ethanol for 3 minutes at RT.
9. Rinse slides gently with running distilled water for 5 minutes at RT.

Antigen Retrieval:

10. Boil slides in 0.01M sodium citrate buffer (pH6) at 100°C for 15-20 minutes. Remove the slides from heat and allow them to stand at RT in buffer for 20 minutes.
11. Rinse twice with Tris-Buffered Saline + Tween (TBST) for 5 minutes at RT.

Immunostaining:

Recommended: Do not allow tissues to dry at any time during the staining procedure.

12. Block endogenous peroxidase with 3% hydrogen peroxide for 30 minutes.
13. Block with 5% serum or BSA for 2 hours at RT.
14. Drain blocking buffer from slide.
15. Incubate slides with the diluted primary antibody overnight at 4°C with gentle agitation.

16. Wash slides 2 times with TBST for 5 minutes at RT.
17. Develop with chromogen for 10 minutes at RT.
18. Wash slides in distilled water for 1 minute at RT.
19. Counterstain (if required).
20. Dehydrate when using a chromogen substrate that is alcohol insoluble by washing slides in 80%, 95%, 100% and Xylene each for 1 minute at RT.
21. Mount coverslips.