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Original Research Article

Immunohistochemical and Morphometric Study of Urinary Bladder Epithelial Lesions with Special References to Invasiveness and Proliferative Activity

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ABSTRACT

Context (background) & aims – The urinary bladder is a part of lower urinary tract, one of the main portals of the urogenital system. The different bladder lesions constitute a major health problem in developing countries. Carcinoma of this region is the commonest neoplasm in elderly male in our country. Our study was done with the aim of early diagnosis of these lesions in a cost effective manner.

Materials & Methods - Total 62 cases were selected having signs and symptoms of lower urinary tract infection and hematuria. The specimens were collected from the Urology department of a tertiary care hospital situated at Kolkata. Thereafter, the tissues were processed routinely for histopathology and immunohistochemistry (p53, ki-67 & 34βE12 cytokeratin).Morphometric analysis of Mean Nuclear Diameter (MND), Mean cytoplasmic diameter (MCD), Mean nuclear area (MNA), Mean Nuclear Perimeter (MNP), Nucleo-cytoplasmic ratio (N: C) etc were done on H & E stained sections with ERMA ocular micrometer and analysed by software AutoCAD 2007.

***Statistical analysis used* -** Unpaired Student's t-Test.

Results - Statistically significant difference was found between non-neoplastic and malignant lesions. The diagnosis was confirmed by histopathological findings along with morphometry and proliferative activities as well as invasiveness were assessed with ki-67 and 34βE12 cytokeratin immunolabelling.

Conclusion - Morphometry can be an important tool for early diagnosis and determination of therapeutic protocol in the different urinary bladder lesions especially if they are correlated with immunohistochemistry.

***Key words* -** Urinary bladder lesion, Morphometry, Immunohistochemistry, ki-67.

INTRODUCTION

The bladder cancer is one of the major problems in India and it is the ninth most common cancer. Incidence is found as 3.9% of all cancer cases as per the Indian Cancer Registry data. [1] Patients with urinary bladder lesions may be asymptomatic and have occult bladder lesions that are detected with routine screening .Sometimes the distinction between premalignant dysplasia and carcinoma in situ or minimally invasive carcinoma is difficult in routine Haematoxylin and Eosin (H & E) stained

sections. The epithelial changes that have some but not all of the features of CIS are most commonly termed dysplasia. The morphology of dysplasia generally is of cohesive cells, with umbrella cells usually present, characterized by mild nuclear / nucleolar changes that focally include irregular nuclear crowding and slight hyperchromasia. Anisonucleosis is generally present; there may be an increased number of cell layers; nucleoli may be prominent; mitotic figures when present are generally basally located. Most cellular abnormalities in dysplasia are restricted to the basal and intermediate layers. [2] In atypia of unknown (uncertain) significance the nuclear changes similar to those seen in reactive atypia. The degree of nuclear pleomorphism and/or hyperchromasia is greater than in the latter and dysplasia cannot always be ruled out with certainty .When morphometry and image analysis were used, it was seen that dysplasia has a pattern similar to that of papillary carcinoma of grade 1 (WHO 1973). [3] Application of immunohisto-chemistry has been found to be an important tool to resolve the problems in microscopic diagnosis of these grey zones in histopathology. The antibody 34βE12 stains selectively the keratins of basal cells; it has been used in the differential diagnoses between different invasive carcinomas and the benign, premalignant and in situ malignant lesions that simulate it. [4] The monoclonal antibody 34βE12 is specific for "high molecular weight" cytokeratin and characteristically found in complex epithelium. The antibody reacts with all squamous and ductal epithelium and stains carcinomas. It reacts with benign small acinar lesions of prostate. [5] 'The benign small acinar lesions in the prostate gland are difficult to differentiate from small acinar adenocarcinoma. An important diagnostic criterion in the differentiation is the loss of basal layer in small acinar adenocarcinoma and its presentation in benign lesions. A monoclonal antibody to high molecular weight cytokeratin (34βE12) has been shown to stain these basal cells peferentially. [6] In this study we have considered the expression of p53 and ki-67 labelling index [LI] as cell proliferative markers and 34βE12 cytokeratin as a marker invasion (basal cells) in urothelial lesions.

Precisely the aims and objectives of our study were -

1) Evaluation of the role of morphometry in early diagnosis of the different urinary bladder lesions.

2) Role of immunohistochemistry in diagnosis of these lesions with assessment of proliferative activity and invasiveness.

MATERIALS AND METHODS

The study was performed in the department of pathology of a tertiary care hospital situated at Kolkata over a period of 2 years. Our study population (62 cases) was the patients attending the outpatient department of urology of this hospital and also those admitted in the urology indoor with persistent hematuria, pain abdomen, frequency, urgency, dysuria, lump or mass in the abdomen etc. Informed consent was taken from the patients or their guardians. At first, each patient was clinically assessed. After going through a detailed history, clinical examinations along with general and systemic examinations were performed to make a provisional diagnosis. Reports of routine blood investigations, radiological investigations and cystoscopic findings were also recorded. The specimens were taken from transurethral resection of bladder tumor (TURBT), open cystectomy etc.

The specimens were examined for gross findings and tissue obtained were fixed in formalin, processed and embedded in paraffin wax block. One section of 3 micron thickness from each block was affixed on egg albumin coated slide for H&E staining and three sections 3 micron thickness each from each block were affixed on poly-L-Lysine coated slides for basal cell high molecular weight cytokeratin (34βE12), Proliferative cell nuclear antigen (ki-67) and p53 study.

The morphometry was done on the urothelial cells in the histological sections

with the aid of an ocular morphometer (ERMA ocular micrometer) attached to the 10X eyepiece of a microscope using a 40X high power objective. The ocular morphometer was calibrated using a calibration slide or stage micrometer provided with it. One small division on the stage micrometer is equated with 0.01 mm. One small division of ERMA ocular micrometer is equated with 2.22µm. 50 random nuclei from grossly cellular atypical smear and 30 random nuclei from rest of the cases were subjected to analysis. [7] The images were also analyzed by using the software AutoCAD 2007. The characteristics studied upon were major and minor axis of cell and nuclei, mean cell diameter, mean nuclear diameter, mean cell area, mean nuclear area as observed and calculated in different smears. The nuclear area (A) was calculated by the formula A = π x a x b, where a and b are the semi-largest and semi-smallest nuclear diameter. Similar formula was applied for cell area. The nucleo-cytoplasmic ratio (N: C) was obtained from dividing nuclear diameter by cell diameter. The sections for controls were taken from the adjacent normal bladder tissues which were sent along with the different masses.

For immunohistochemical staining, the kit literature of the manufacturer was followed.

H&E stained slides were examined thoroughly and a diagnosis of each case was made. p53 staining was evaluated by the positive cases (the percentage of cases showing positive staining) and p53 positivity was assessed by the percentage of nucleus showing positive staining out of total nuclei counted (300-1000). ki-67 staining was evaluated by ki-67 labeling index i.e. ki-67 LI % - the percentage of nucleus showing positive staining out of total nuclei counted (500-1000) and degree of invasiveness was assessed by staining with 34βE12 as it stains selectively the basal cells. Finally a grand chart was produced tabulating histological diagnosis, cytokeratin expression, p53 nuclear positivity and ki-67LI %. Statistical analysis was done by Unpaired Student's 't' test and P values were obtained. A level of significance of 5% (p value <0.05) is chosen, for no better reason than that it is conventional.

RESULTS AND ANALYSIS

We studied total 62 cases in the department of Pathology, IPGME&R, Kolkata, in the period of 2 years. Our study population was the patients attending in urology OPD of a tertiary care hospital of Kolkata. Diagram 1 showed distribution of cases depending on the histopathological diagnosis (n=62). . In our study the most common benign lesion of bladder was found to be cystitis whereas low grade TCC was the most common malignant lesion of bladder. Most of the benign lesions such as cystitis cases were found in 20-40 yrs of age group. In case of low grade TCC most of the patients were found to be in the age group of 41-60 years, whereas most of the cases of high grade TCC were found in the age group of 61-80 years which is definitely higher than the previous age group. In case of adenocarcinoma most of the patients were 41-60 years of age, whereas most of the cases of SCC were found to be in the age group of 61-80 years.

Reactive process like cystitis cases mostly seen in female and most of the neoplastic lesions more commonly found in male except adenocarcinomas which shows female preponderance. The inflammatory lesions of bladder like cystitis were presented with the complain of abdominal pain and dysuria but hematuria was found to be the most common symptom in neoplastic lesions.

Total 62 cases were included in this study. All of them were diagnosed on routine H & E stained sections. Diagram 1 described the distribution of cases according to histopathological diagnosis. Morphometric analysis were done on routine H & E stained sections in terms of mean nuclear diameter (MND), mean nuclear area (MNA), mean cell diameter

(MCD), mean cell area (MCA), nucleocytoplasmic ratio (N:C) etc and the results are shown in Table 1.Statistical analysis showed from Table 2,3,4,5 there is no significant difference in MND and MNA between control and cystitis, as cystitis has low proliferative activity. In case of adenocarcinoma and SCC both have high proliferative activity but there is no significant difference in MND and MNA of these lesions. Although there is significant difference in these parameters between reactive and malignant lesions. Though the cystitis has low proliferative activity but there is significant difference in MCD and MCA between control and cystitis. Whereas in case of adenocarcinoma and SCC both have high proliferative activity but there is no significant difference in MCD and MCA of these lesions. Although there is significant difference in these parameters between reactive and malignant lesions of bladder.

The inflammatory lesion such as cystitis has low proliferative activity but there is significant difference in N/C between control and cystitis which is shown in Table 10. Whereas in case of adenocarcinoma and SCC both have high proliferative activity but there is no significant difference in N/C of these lesions. [Table 6]Although there is significant difference in these parameters between reactive and malignant tumors.

We studied the proliferative activity of different urinary bladder lesions with immunohistochemistry using monoclonal antibody against P53 and ki-67 and the results are shown in Table 7 and 8. A tumor with positive nuclear staining in more than 20% of tumor cells were interpreted as positive expression of p53.

As the cystitis has low proliferative activity, there is no significant difference in ki-67 LI between control and cystitis which is shown in Table 9. Whereas in case of adenocarcinoma and SCC both have high proliferative activity but there is no significant difference in PCNA LI of these lesions. Although there is significant difference in these parameters between benign and malignant lesions.

We studied the invasiveness of different urinary bladder lesions by immunohistochemistry with 34βE12 expression which is shown in Table 10. The continuous pattern of staining was seen in control and benign lesions whereas discontinuous pattern was seen in malignant conditions.

PHOTOMICROGRAPHS

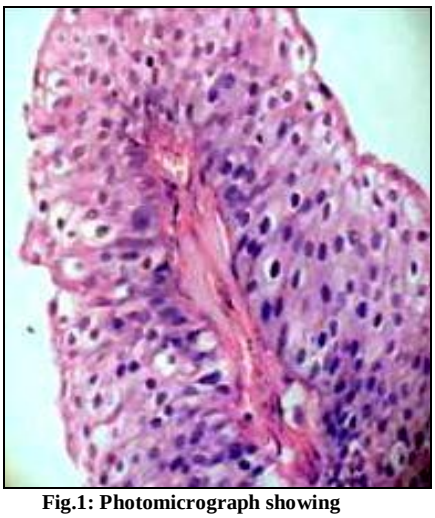

Fig.1: Photomicrograph showing low grade TCC (H&Ex400)

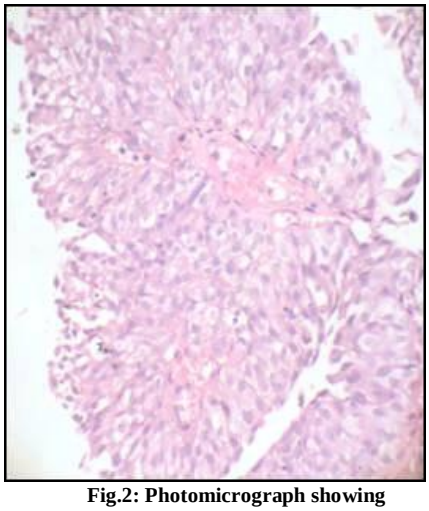

Fig.2: Photomicrograph showing high grade TCC (H&Ex400)

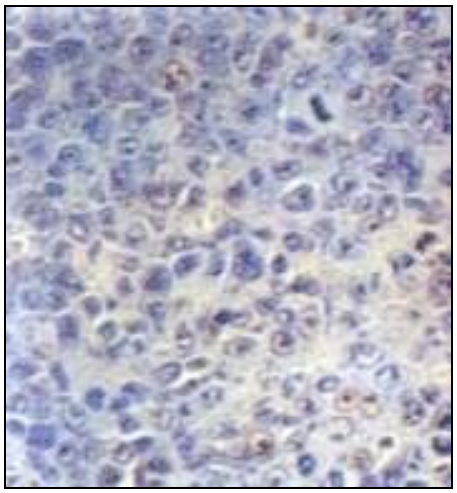

Fig.3: Photomicrograph showing Low grade TCC (Monoclonal antibody against Ki-67x400).

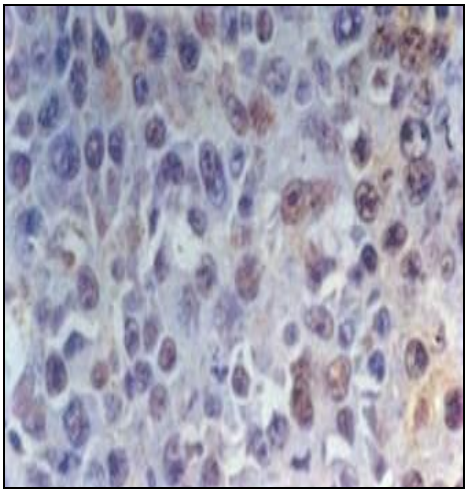

Fig.4: Photomicrograph showing high grade TCC (Monoclonal antibody against Ki-67 x400).

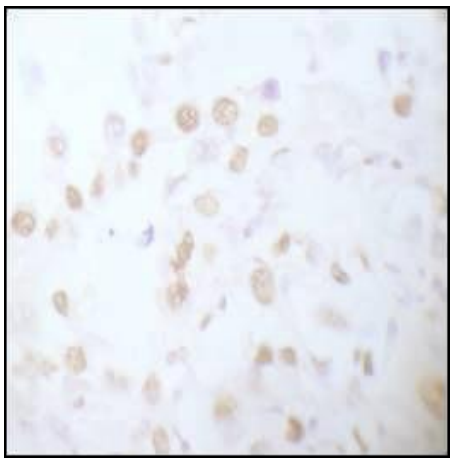

Fig.5: Photomicrograph showing high grade TCC (Monoclonal antibody against p53 x400).

Fig.6: Photomicrograph showing high grade TCC (Monoclonal antibody against 34βE12 x400).

RESULTS

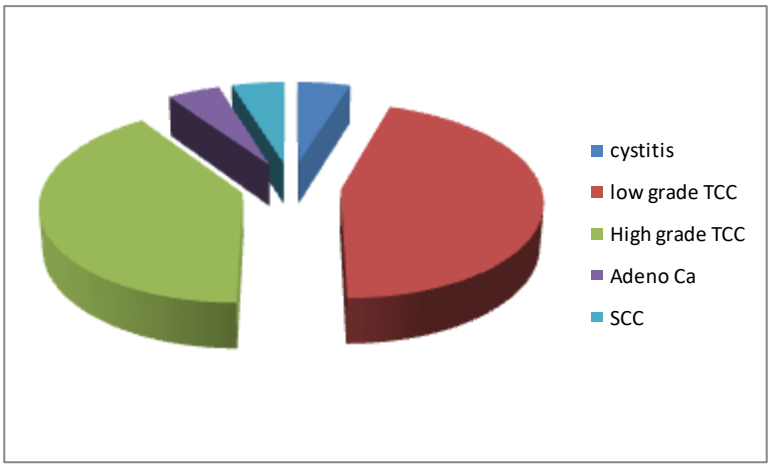

Diagram 1: Distribution of cases depending on the histopathological diagnosis (n=62)

Table 1: Results of morphometric study (on H & E stained sections) (n=62).

Histopathological Diagnosis	MND ± 2SD µm	MNA ± 2SD µm^2	MCD ± 2SD µm	MCA ± 2SD µm^2	MND:MCD ± 2SD
Control	8.053±0.092	50.962±1.173	10.86±0.052	92.667±0.902	0.741±0.006
Cystitis	8.073±0.064	51.213±0.814	10.16±0.04	82.185±2.443	0.794±0.008
Low Grade TCC	8.893±0.056	62.148±0.787	10.727±0.087	90.43±1.474	0.828±0.006
High Grade TCC	9.389±0.19	69.301±2.794	10.48±0.213	86.86±2.971	0.895±0.006
Adeno CA	15.227±2.616	185.77±59.546	18.062±3.728	263.629±99.628	0.847±0.034
SCC	17.278±0.068	234.578±1.848	20.274±0.238	323.006±7.562	0.852±0.007

Table 2: p-values showing the significance of differences in MND between lesions

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	0.773 (NS)	$2.775X10^{-20}$ (S)	$5.58X10^{-12}$ (S)	0.008 (S)	$1.597X10^{-8}$ (S)
Cystitis	0.773 (NS)	-	$1.571X10^{-20}$ (S)	$6.97X10^{-12}$ (S)	0.009 (S)	$7.153X10^{-9}$ (S)
Low Grade TCC	$2.775X10^{-20}$ (S)	$1.571X10^{-20}$ (S)	-	$4.795X10^{-18}$ (S)	$2.709X10^{-15}$ (S)	$1.953X10^{-49}$ (S)
High Grade TCC	$5.58X10^{-12}$ (S)	$6.97X10^{-12}$ (S)	$4.795X10^{-18}$ (S)	-	$1.051X10^{-12}$ (S)	$3.516X10^{-31}$ (S)
Adeno CA	0.008 (S)	0.009 (S)	$2.709X10^{-15}$ (S)	$1.051X10^{-12}$ (S)	-	0.246 (NS)
SCC	$1.597X10^{-8}$ (S)	$7.153X10^{-9}$ (S)	$1.953X10^{-49}$ (S)	$3.516X10^{-31}$ (S)	0.246 (NS)	-

Table 3 : p-values showing showing significance of differences in MNA between lesions.

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	0.775 (NS)	$7.034X10^{-20}$ (S)	$2.324X10^{-11}$ (S)	0.0172 (S)	$1.347X10^{-8}$ (S)
Cystitis	0.775 (NS)	-	$4.62X10^{-20}$ (S)	$2.885X10^{-11}$ (S)	0.0173 (S)	$9.813X10^{-9}$ (S)
Low Grade TCC	$7.034X10^{-20}$ (S)	$4.62X10^{-20}$ (S)	-	$8.399X10^{-18}$ (S)	$1.27X10^{-13}$ (S)	$8.562X10^{-53}$ (S)
High Grade TCC	$2.324X10^{-11}$ (S)	$2.885X10^{-11}$ (S)	$8.399X10^{-18}$ (S)	-	$1.313X10^{-11}$ (S)	$4.887X10^{-35}$ (S)
Adeno CA	0.0172 (S)	0.0173 (S)	$1.27X10^{-13}$ (S)	$1.313X10^{-11}$ (S)	-	0.228 (NS)
SCC	$1.347X10^{-8}$ (S)	$9.813X10^{-9}$ (S)	$8.562X10^{-53}$ (S)	$4.887X10^{-35}$ (S)	0.228 (NS)	-

Table 4: p-values showing the significance of differences in MCD lesions.

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	$5.269X10^{-5}$ (S)	0.0166 (S)	0.005 (S)	0.028 (S)	$3.002X10^{-7}$ (S)
Cystitis	$5.269X10^{-5}$ (S)	-	$7.46X10^{-12}$ (S)	0.016 (S)	0.021 (S)	$2.164X10^{-7}$ (S)
Low Grade TCC	0.0166 (S)	$7.46X10^{-12}$ (S)	-	$7.718X10^{-7}$ (S)	$5.177X10^{-13}$ (S)	$1.963X10^{-43}$ (S)
High Grade TCC	0.005 (S)	0.016 (S)	$7.718X10^{-7}$ (S)	-	$6.426X10^{-12}$ (S)	$7.634X10^{-32}$ (S)
Adeno CA	0.028 (S)	0.021 (S)	$5.177X10^{-13}$ (S)	$6.426X10^{-12}$ (S)	-	0.363 (NS)
SCC	$3.002X10^{-7}$ (S)	$2.164X10^{-7}$ (S)	$1.963X10^{-43}$ (S)	$7.634X10^{-32}$ (S)	0.363 (NS)	-

Table 5; p-values showing the significance of differences in MCA lesions.

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	0.0022 (S)	0.016 (S)	0.0026 (S)	0.041 (S)	$7.949X10^{-7}$ (S)
Cystitis	0.0022 (S)	-	$1.41X10^{-9}$ (S)	0.014 (S)	0.034 (S)	$7.888X10^{-7}$ (S)
Low Grade TCC	0.016 (S)	$1.41X10^{-9}$ (S)	-	$7.651X10^{-7}$ (S)	$9.399X10^{-12}$ (S)	$5.079X10^{-44}$ (S)
High Grade TCC	0.0026 (S)	0.014 (S)	$7.651X10^{-7}$ (S)	-	$9.039X10^{-11}$ (S)	$3.389X10^{-36}$ (S)
Adeno CA	0.041 (S)	0.034 (S)	$9.399X10^{-12}$ (S)	$9.039X10^{-11}$ (S)	-	0.361 (NS)
SCC	$7.949X10^{-7}$ (S)	$7.888X10^{-7}$ (S)	$5.079X10^{-44}$ (S)	$3.389X10^{-36}$ (S)	0.361 (NS)	-

Table 6: p-values showing the significance of differences in N/C ratio between lesions.

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	0.001 (S)	$1.921X10^{-19}$ (S)	$4.686X10^{-24}$ (S)	0.006 (S)	$3.656X10^{-5}$ (S)
Cystitis	0.001 (S)	-	$4.367X10^{-9}$ (S)	$4.4X10^{-19}$ (S)	0.043 (S)	0.0009 (S)
Low Grade TCC	$1.921X10^{-19}$ (S)	$4.367X10^{-9}$ (S)	-	$6.436X10^{-38}$ (S)	0.008 (S)	$3.356X10^{-6}$ (S)
High Grade TCC	$4.686X10^{-24}$ (S)	$4.4X10^{-19}$ (S)	$6.436X10^{-38}$ (S)	-	$3.231X10^{-7}$ (S)	$9.207X10^{-11}$ (S)
Adeno CA	0.006 (S)	0.043 (S)	0.008 (S)	$3.231X10^{-7}$ (S)	-	0.839 (NS)
SCC	$3.656X10^{-5}$ (S)	0.0009 (S)	$3.356X10^{-6}$ (S)	$9.207X10^{-11}$ (S)	0.839 (NS)	-

Table 7: ki-67 Labeling Index of different lesions.

Histopathological Diagnosis	Range of PCNA LI (%)	Mean of PCNA LI (%)
Control	3-5	4
Cystitis	4-6	5
Low Grade TCC	28-40	34.5
High Grade TCC	55-75	65.2
Adeno CA	54-60	57.33
SCC	51-55	53.33

Table 8: p-values showing the significance of differences in ki-67 LI between lesions.

Histopathological Diagnosis	Control	Cystitis	Low Grade TCC	High Grade TCC	Adeno CA	SCC
Control	-	0.287 (NS)	$2.104X10^{-14}$ (S)	$1.789X10^{-16}$ (S)	$8.727X10^{-6}$ (S)	$3.185X10^{-6}$ (S)
Cystitis	0.287 (NS)	-	$4.884X10^{-14}$ (S)	$2.665X10^{-16}$ (S)	$9.411X10^{-6}$ (S)	$3.457X10^{-6}$ (S)
Low Grade TCC	$2.104X10^{-14}$ (S)	$4.884X10^{-14}$ (S)	-	$3.76X10^{-29}$ (S)	$3.952X10^{-11}$ (S)	$2.053X10^{-9}$ (S)
High Grade TCC	$1.789X10^{-16}$ (S)	$2.665X10^{-16}$ (S)	$3.76X10^{-29}$ (S)	-	0.026 (S)	0.001 (S)
Adeno CA	$8.727X10^{-6}$ (S)	$9.411X10^{-6}$ (S)	$3.952X10^{-11}$ (S)	0.026 (S)	-	0.134 (NS)
SCC	$3.185X10^{-6}$ (S)	$3.457X10^{-6}$ (S)	$2.053X10^{-9}$ (S)	0.001 (S)	0.134 (NS)	-

[S]= Significant; [NS] = Not significant. ; p value < 0.05 is considered as significant.

Table 9: P53 Expression in different lesions.

Histopathological Diagnosis	Negative (-Ve)	Positive (+Ve)
Control (n=3)	3 (100%)	0
Cystitis (n=3)	3 (100%)	0
Low Grade TCC (n=28)	11 (39.28%)	17 (60.72%)
High Grade TCC (n=25)	5 (20%)	20 (80%)
Adeno CA (n=3)	0	3 (100%)
SCC (n=3)	0	3 (100%)

Table 10: 34βE12 expression in different lesions.

Histopathological Diagnosis	34βE12 expression
Control (n=3)	Continuous
Cystitis (n=3)	Continuous
Low Grade TCC (n=28)	Discontinuous
High Grade TCC (n=25)	Discontinuous
Adeno CA (n=3)	Discontinuous
SCC (n=3)	Discontinuous

DISCUSSION

The histological sections were taken from all the lesions and the sections for controls were taken from the adjacent normal bladder tissues which were sent along with the different masses. All the cases were diagnosed with H & E stained sections. All the benign lesions of urinary bladder in our study (3 cases) are cystitis. Among 59 malignant lesions of urinary bladder found in our study- 53 cases are transitional cell carcinomas [low grade- 28 (Fig. 1) and high grade- 25 (Fig. 2)] and 3 each of adenocarcinomas and squamous cell carcinomas (Table 1). The transitional cell carcinomas of urinary bladder were graded microscopically as per WHO system. We have found in our study that the cystitis cases were found in 20-40 yrs of age group and low grade TCC are mostly within the age group of 41-60 yrs. whereas most of the cases of high grade TCC were found in the age group of 61-80 years. The reactive process like cystitis mostly seen in female and most of the neoplastic lesions more commonly found in male except adenocarcinomas which shows female preponderance. Cystitis is particularly common in young women of reproductive age and in older age groups of both sexes. [8] About 80% of patients are between the ages of 50 and 80 years. The male to female ratio for urothelial tumors is approximately 3:1. [9] The inflammatory lesions of bladder like cystitis were presented with the complain of abdominal pain and dysuria but hematuria was found to be the most common symptom in neoplastic lesions. All form of cystitis are characterized by a triad of symptoms like frequency, lower abdominal pain and dysuria. [10] But in case of neoplastic lesions of bladder gross or microscopic hematuria is the most common form of presentation, followed by symptoms related to associated urinary tract infection. [11] Morphometric analysis was performed with H & E stained histological sections in terms of mean nuclear diameter (MND), mean nuclear area (MNA), mean cell diameter (MCD), mean cell area (MCA), nucleocytoplasmic ratio (N:C) etc.

In this study, MND of controls were found to be 8.053±0.092 µm and that of cystitis 8.073±0.064 µm and in malignant lesions such as low grade TCC, high grade TCC, adenocarcinomas and SCC were found to be 8.893±0.056 µm, 9.389±0.19 µm, 15.227±2.616 µm and 17.278±0.068 µm respectively (Table 1). In our study, MNA of controls were found to be 50.962±1.173 μm^2 and that of cystitis 51.213±0.814 μm^2 and in malignant lesions like low grade TCC, high grade TCC, adenocarcinomas and SCC were found to be 62.148±0.787 μm^2, 69.301±2.794 μm^2, 185.77±59.546 μm^2 and 234.578±1.848 μm^2 respectively (Table 1). Thus MNA of TCC cases were found to be gradually increasing with advancement of the

histological grade of the lesions and certainly the values of malignant lesions are higher than that of the controls as well as that of the inflammatory lesions. Ramos D et al. studied value of morphometry in low grade papillary urothelial bladder neoplasms and they found MNA of the 10 largest nuclei was > or = 52 μm^2. [12] Fukuzawa S et al. examined 161 patients with untreated bladder carcinomas and nuclear morphometric values were measured on each subject and they found that survival rates were significantly lower among patients with bladder carcinomas where MNA > or = 33.6 μm^2. [13] In this study, MCD of controls, cystitis, low grade TCC, high grade TCC, adenocarcinomas and SCC were found to be 10.86±0.052 µm, 10.16±0.04 µm, 10.727±0.087 µm, 10.48±0.213 µm, 18.062±3.728 µm and 20.274±0.238 µm respectively and MCA were found to be 92.667±0.902 μm^2, 82.185±2.443 μm^2, 90.43±1.474 μm^2, 86.86±2.971 μm^2, 263.629±99.628 μm^2 and 323.006±7.562 μm^2 (Table 1). Shunsuke Hanai, M.D. et al. found that the cytoplasmic area was more in normal epithelium than transitional cell carcinoma and the cytoplasmic area gradually decreasing with advancement of the histological grade of TCC. In our study MCA of normal transitional epithelial was higher than that of TCC and MCA of low grade TCC was also than that of high grade TCC. [14]

The N/C ratios of controls (0.741±0.006) and cystitis (0.794±0.008) were found to be lower than that of the malignant lesions. We found an increase in N/C ratio of TCC with advancement of the histological grade from low grade (0.828±0.006) to high grade (0.895±0.006). N/C ratios of adenocarcinomas (0.847±0.034) and SCC (0.852±0.007) were also higher than that of cystitis. Shunsuke Hanai, M.D. et al. analyzed the cytomorphometrical study of cells of carcinoma in situ had the characteristics of grade II or grade III carcinoma cells. The morphometrical features of the advanced cases of grade II carcinoma revealed that the nuclear area and the cytoplasmic area were rather small, whereas the N/C ratio was almost average for the grade II group. [14]

Unpaired Student's t-Test was done to calculate the significance of difference in different parameters of bladder lesions (p value <0.05). MND and MNA can be used as an important morphometric parameter to differentiate normal epithelial cells and cystitis from different malignant bladder lesions like TCC, adenocarcinomas and SCC etc. because the difference in their MND and MNA values were found to be statistically significant (p <0.05). (Table 2 and 3) The differences between MND and MNA values of controls and cystitis were not found to be statistically significant as cystitis has low proliferative activity. So we concluded that it is not so useful parameter for differentiating cystitis from the normal transitional epithelium. The p value of both the parameters was found to be significant between different grades of TCC. Shunsuke Hanai, M.D. et al. found that there were significant differences ($p < 0.05$) in nuclear area between normal epithelium obtained by TUR and grade I transitional cell carcinoma. The morphometrical features of the advanced cases of grade II carcinoma revealed that the standard deviation of the nuclear area were small than grade I TCC. [14]

In this study, MCD and MCA were found to be significant in differentiating controls and reactive lesions as well as all the malignant lesions like TCC, adenocarcinomas and SCC etc. (Table 4 and 5) The N/C ratios of various bladder lesions were found a gradual increase through normal epithelial cells to reactive lesions and finally to malignant lesions. The p values were found to be highly significant in differentiating controls and cystitis as well as various malignant lesions of bladder. Again we found the higher value of N/C ratio in high grade TCC than that of low grade TCC and the difference was statistically significant. (Table 6) Shunsuke Hanai, M.D. et al. found that there were

significant differences N/C ratio between normal transitional epithelium and grade I carcinoma ($p < 0.05$). [14]

From the study of various morphometric parameters, we finally concluded that nuclear and cytoplasmic parameters were found to be significant in differentiating various bladder lesions because both the parameters can differentiate normal epithelium and neoplastic cells. Kapur U. et al. told from their study that quantitative nuclear morphometry could aid in the objective grading of urinary bladder biopsies. This information might aid the treating physicians in better risk stratification of patients with urothelial carcinoma. [15]

We studied the proliferative activity of the lesions with immunohistochemistry using monoclonal antibody against P53 and ki-67. In our study the range of ki-67 LI values in controls found to be 3-5% with a mean 4% and that of cystitis 4-6% (mean 5%) and in malignant lesions like low grade TCC, high grade TCC, adenocarcinomas and SCC were found to be 28-40% (mean 34.5%), 55-75% (mean 65.2%), 54-60% (mean 57.33%) and 51-55% (mean 53.33%) respectively. (Table 7) As the cystitis has low proliferative activity, there is no significant difference in ki-67 LI between control and cystitis which is shown in Table 8. Whereas in case of adenocarcinoma and SCC both have high proliferative activity but there is no significant difference in ki-67 LI of these lesions. Although there is significant difference in these parameters between benign and malignant lesions. (Fig. 2 and 3) In a similar study conducted by Sangwan M et al in 2015, morphometry in combination with Ki–67 labelling was found to have high reproducibility in differentiating low and high grade, and visualization of proliferating cells simultaneously. They suggested the use of these multivariate grading model in routine grading to overcome interobserver variability and also to increase reproducibility of grading system for urinary bladder cancer. [16]

In another similar study, significant positive association was found between Ki-67 over expression and stage, grade, metastasis. P value calculated was 0.001, 0.040., and 0.036, respectively. On further follow up, disease recurred in 56% of patients. Kaplan-Meier analyses revealed that Ki-67 overexpression was significantly associated with an increased probability of disease recurrence and bladder cancer-specific mortality in those patients. [17] In our study, a tumor with positive nuclear staining in more than 20% of tumor cells were interpreted as positive expression of p53 and the results are shown in (Table 9). In our study, all the controls and cystitis were negative for p53 staining. We also found that 39.28% of low grade TCC and 20% of high grade TCC were negative for p53 expression. While 60.72% of low grade TCC, 80% of high grade TCC (Fig. 5), all cases of adenocarcinoma and SCC were found to be positive p53 expression. The positive p53 expression of TCC cases were found to be gradually increasing with advancement of the histological grade of TCC. Monireh Halimi et al. reported that positive p53 staining was in 10-75% of low-grade tumors and in more than 58% of high-grade tumors. Ye et al. have reported 40.8% and 78% p53 protein positive staining in low- and high-grade tumors, respectively. Most of the studies have demonstrated that the rate of p53 expression in the patients with high-grade tumor is higher than patients with low-grade tumor. Nonneoplastic urothelium showed no reactivity to p53. [18] B. Kishore et al observed that, out of 110 cases of urinary bladder carcinoma, 84 cases (76.4%) showed high p53 expression, 21 cases (19.0%) showed low p53 expression, and 5 cases (4.6%) were immunonegative for p53. The difference of p53 expression was found to be statistically significant ($P = 0.0001$; df = 1 while taking grading into account .They also observed that mean expression of Ki-67 was consistently increasing higher with staging as compared to p53 expression as well as the difference between expression of

these two markers in pTa and pT1 stage (*P* = 0.0001) as compared to pT2. They also recommend combined use of p53 and Ki-67 immunostaining as prognostic marker along with histological grading and staging. [19]

However, a high p53 expression value might be an indicator for tumor progression or early failure of local therapy warranting early surgical intervention.

The invasiveness of different urinary bladder lesions were studied with 34βE12 expression. (Table10, Fig.6). The continuous pattern of staining was seen in control and benign lesions whereas discontinuous pattern was seen in malignant conditions. The monoclonal antibody 34βE12 is specific for "high molecular weight" cytokeratin and characteristically found in complex epithelium. The antibody reacts with all squamous and ductal epithelium and stains carcinomas. It reacts in continuous pattern in case of benign lesions whereas discontinuous pattern in case of carcinomas regardless of grade of tumors. [11] Green et al. found immunohistochemical stains for (IHC) high molecular weight cytokeratin (HMWCK) may show the presence or absence of basal cells in a benign or malignant diagnosis respectively. [20] Chitale A, Khubchandani S et al. found that basal cell hyperplasia consists of a proliferation of basal cells in two or many layers near the basement membrane of prostatic acini. These cells are immunoreactive for high molecular weight cytokeratin (34βE12) and this will distinguish the lesion from adenocarcinoma. [21]

Diagnosis of frank benign or malignant lesions usually do not pose problem in histopathology but difficulty arises in differentiating between the premalignant lesions like papillary urothelial hyperplasia, flat hyperplasia, atypia of unknown significance, urothelial dysplasia, low-grade intraurothelial neoplasm etc. Morphometry and immunohistochemistry (IHC) can solve this problem.

CONCLUSION

So, we can conclude that the morphometric analysis of histological sections increases the effectivity of histology but immunohistochemistry remains the gold standard for diagnosis of proliferative activity and invasiveness of urinary bladder lesions.

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