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ROLE OF P 53 AND KI-67 AS A PROGNOSTIC BIOMARKER OF UROTHELIAL CARCINOMA.

Oncopathology

Satotsna Patra MD Assistant Professor, Department of Pathology, Hi-Tech Medical College and Hospital, Odisha, India.

Ruchi Mittal MD, DNB Professor, Department of Pathology, Kalinga Institute of Medical Sciences, Bhubaneswar, Odisha, India.

Anubhav Abinash Sahu MD Assistant Professor, Department of Pathology, SCB Medical College & Hospital, Cuttack, Odisha, India.

Sunil Agarwal MD Professor, Department of Pathology, Hi-Tech Medical College and Hospital, Odisha, India.

Liza Das* MD Tutor, Department of Pathology, Shree Jagannath Medical College & Hospital, Puri, Odisha, India. *Corresponding Author

Raghumani Mohanty MD Department of Pathology, Hi-Tech Medical College and Hospital, Odisha, India.

ABSTRACT

Introduction: Bladder cancers are common malignancies worldwide, and histological examination is the cornerstone of diagnosis and management of these tumours. There exists huge interobserver variability in grading of urothelial carcinoma, furthermore superficial biopsies and cautery effect also effect tumour staging. Pathological grading, staging and their correlation with molecular markers like p53 and Ki-67 might better predict patient outcome and play significant role in designing the management protocols of these patients. Thus, we aimed to study the role of p 53 and Ki-67 as prognostic and diagnostic biomarkers of urothelial carcinoma in south east Indian population. **Methods:** This was a prospective study that included 46 patients with Urothelial carcinoma of the urinary bladder. The histopathological evaluation of the tumor tissue was performed to assess the grade and stage of the tumor and was further correlated with important demographic details and with immunohistochemical expression of p 53 and Ki-67. **Results:** The mean age was 55 and 65 years for male and female patients respectively. Haematuria was the most common presenting symptom. A total of 38 (74%) patients had high-grade tumors. p T2 stage disease was observed in 60% patients. High expression of p 53 expression was noted in 52% of total cases, of which 85% of cases had high grade morphology and 66% were p T2 stage tumours. High expression of p 53 was also noted in 45% of p T1 tumors. Ki-67 showed high expression in 82.6 % of cases; 94% of which were high-grade and 25% were low-grade cases. **Conclusion:** Although males have preponderance for affliction, but in our country, females also show higher incidence of urothelial carcinoma as compared to published literature. In addition, patients in Indian population present with higher stage disease. p 53 and Ki-67 are biomarkers of higher stage and grade disease, however additional factors also play a definite role in tumour progression and thus in patient outcome.

KEYWORDS

INTRODUCTION:

Bladder neoplasms are the 5th most common malignancies worldwide [1] its' incidence being four times higher in males than in females.[2] As per the Indian cancer registry data, it is the 9th most common cancer accounting for 3.9% of all cancer cases[3] In India, according to the recent reports of the National Cancer Registry Programme, the overall incidence rate of the urinary bladder cancer is 2.25% (per 100,000 annually): 3.67% for males and 0.83% for females.[4]

Many genetic polymorphisms are identified in bladder cancer etiopathogenesis which influence susceptibility, prognosis or therapy response in bladder tumors.[5] Infectious and inflammatory disorders also promote tumor development and rapid progression of carcinogenetic process. Risk factors for bladder neoplasm include smoking, family history, prior radiation therapy, frequent bladder infections, and exposure to certain chemicals. [6]

The most common type of bladder neoplasm is transitional cell carcinoma(90%), other types include squamous cell carcinoma and adenocarcinoma(10%)[6] .Prognosis of even a high grade TCC is not essentially poor, but in presence of extensive involvement and variants like squamous cell carcinoma or adenocarcinoma confers a worse prognosis. Low grade tumors have a 10 year survival of 98%, however it is less than 50% in high grade invasive carcinomas.

Hematuria is the presenting symptom in 90% of patients with bladder cancer, other symptoms may include urinary frequency, urgency, dysuria, and ureteral obstruction. Systemic manifestations indicate metastatic disease and portend a poor prognosis.[7, 8] Diagnosis is typically by cystoscopy with tissue biopsies. Staging of the cancer is determined by medical imaging such as MRI, CT scan and bone scan along with histopathological grading and staging.

Well-established prognostic factors for bladder cancer include tumor stage and grade. Histological tumor grade is considered as an important prognostic factor, particularly for non-muscle-invasive bladder cancer.[9] Tumor stage also has an association with the prognosis of bladder cancer. In one study, the 5-year recurrence-free survival is 76 % in patients with pT1 tumors, 74 % in pT2, 52 % in pT3, and 36 % in pT4 tumors. [10] Tumor stage and nodal involvement have been proven to be the only independent predictors of muscle invasive bladder cancer survival [11]

To prevent rise in mortality, early detection, accurate staging and grading by histopathological examination and immunoprofile of bladder tissue remains the cornerstone, however grading of urothelial carcinoma is associated with significant interobserver variability even to the experienced pathologists. Superficial biopsies and cautery effect also poses challenges in appropriate tumour staging, specifically in general hospitals.

In such a scenario correlation of histopathological grading and staging with immunohistochemical markers such as Ki-67 and P53 can help define a group of cases who might have poor prognosis.

p53 is the most frequently mutated tumor suppressor gene identified in urothelial cancers.[12] p53 induces cell cycle arrest in late G1 and S phase which initiates apoptosis in response to genotoxic stress and hypoxia, and mutational inactivation of p53 is associated with an increased risk of tumorigenesis [13]. Alterations in the p53 pathway contribute to bladder tumor progression and are likely to provide relevant prognostic information to assist in the targeted management of bladder cancer patients [14].

The aim of our study was to evaluate the cases of urothelial carcinoma

in a tertiary care centre in Odisha and to study the clinicopathological characteristics and highlight the utility of ki-67 and p53 as prognostic markers to assist the clinician for proper management of cases and also to demonstrate the correlation of ki-67 and p53 immuno-expression with grade and stage of bladder carcinomas at the first presentation, so that it can be included as a standard diagnostic test to assist management and predict patient outcome.

METHODS:

This was a prospective study carried out in the Department of Pathology in collaboration with the Department of Urology. The study extended over a period of 2 years from October 2016 to November 2018. All patients irrespective of the age & gender who attended the outdoor clinics and were admitted to Department of Urology with the clinical suspicion of bladder neoplasm were included in our study. Patients with a prior history of non-neoplastic lesions of the bladder or patients with earlier diagnosis of carcinoma bladder with recurrence during or after treatment were excluded. Informed consent was taken from all the patients and the study adhered to the Declarations of Helsinki. The study was approved by the Institutional Ethics Committee.

Relevant clinical data including history, demographic details, clinical and investigational data were obtained from the patients as well as from the archived case records. All data were recorded in a carefully structured proforma. The laboratory investigations included complete blood count, KUB X – ray, IVP, USG of pelvis and CT scan if deemed necessary. Cystoscopic findings were also recorded. The samples for biopsy included transurethral resection of bladder tumours (TURBT). The biopsy was considered as adequate if it included multiple bits of tissue from the tumor and adjacent mucosa including the muscularis propria.

The biopsy specimens were obtained from the operation theatre in labelled containers using 10% neutral buffered formaldehyde as the fixative. The sample received was examined macroscopically as regards the size, shape, haemorrhage, necrosis or any other gross features. After 24 hrs of fixation, tissue sections of 5 mm thickness were taken from the appropriate representative sites of the tumor mass and were processed in automated tissue processor. The paraffin blocks were prepared using an automatic tissue embedder and 4-5 µm thick sections were cut by using rotary microtome. The sections were fixed to the glass slide previously coated with Meyer's egg albumin. Subsequently the sections were stained by routine hematoxylin and eosin stain. The sections were evaluated independently by 2 senior pathologists and in cases with discordance, slides were reviewed together to reach a consensus. The parameters recorded included histological type, grade, extent / depth of invasion, lympho-vascular invasion and status of adjacent mucosa if it was present in the specimen.

Immunohistochemical stains were performed on 3-4µm thickness sections mounted on Poly-L-lysine coated slides, purchased from Biogenex. The antigen retrieval was done using TRIS-EDTA Buffer at pH of 9.0 The primary antibodies used included Ki-67(BGX-297, mouse monoclonal antibody in Phosphate buffered saline) and p-53 (ready to use, BGX-53, mouse monoclonal antibody in PBS). Diamino benzidine solution was prepared fresh before use, using 20µl of DAB and 1 ml of substrate buffer. Appropriate positive and negative controls were taken, positive controls included lymph node sections for Ki-67 and tonsillar tissue sections for p53

Immunohistochemical staining was conducted and the proportion of the malignant cells staining positive for the nuclear antigen Ki-67 was evaluated in a quantitative and visual way using light microscope. Low-power magnification (10x) was used to determine areas with the highest number of positive nuclei (hot-spots) within the invasive component and the interpretation was made by calculating the amount of stained cell from 1000 tumour cells, with cut-off point 20%. Less than 20% nuclear expression was counted as "low expression" and >20% count as "high-expression"[15].

Nuclear positivity was seen as dark brown color on a bluish background. Percentage of immunopositive cells was calculated by counting at least 1000 tumor cells in areas of maximum positivity. The results were interpreted taking the cut off value as 20% and divided into three categories as immunonegative, <20% as low, and >20% as a high expression [16].

RESULTS

Our study included 46 cases of bladder neoplasms, their age and gender distribution is tabulated in **Table 1**.

In our study the median age for male was 55 yrs and female was 65yrs with a male to female ratio of 2.83:1.0.

Out of a total number of 46 cases, there were 42 cases who presented with clinical features of haematuria, 3 cases with urinary symptoms (urgency, frequency, dysuria) and 1 case presented with lower abdominal pain.

Out of a total number of 46 cases, 38(73.90%) cases were high grade and 8(26.10%) cases showed features of low grade urothelial carcinoma(Ca). The distribution of cases according to grade and gender is shown in **Figure 1.**

In our study, 73.6 % of high grade carcinoma of bladder (**Figure 2 A, B)** was seen in males and 26.3 % of cases in females. The classification and the staging were performed according to the AJCC-TNM Pathologic Staging of Urinary Bladder Carcinomas (2017). According to the staging there were 8(17.39%) cases in stage p Ta, 11(23.91%) in stage p T1 and 28(60.86%) in stage p T2. The distribution of cases according to stage and gender is tabulated in **Table 2.**

It was observed that 24/46 cases (52.17%) showed high p53 expression (**Figure 2 C**), 18 (39.13%) showed low p53 expression, and 4 (8.69%) were negative for p53. High expression of p53 was seen commonly in high grade tumors (23 cases) as compared to only a single low-grade carcinoma (**Figure 3 A, B)**. Low expression (**Figure 3 C**) was noticed in 39.13% cases; 3 with low grade and 15(32.60%) with high grade morphology. *This difference of p53 expression was statistically significant (P=0.00001) while taking grade into account.*

As shown in **Figure 4**, maximum number of patients with high expression of p53 (18 cases) were observed in pT2 stage as compared to pT1(5 cases) and pTa (1 case;2.17%). All 4(8.69%) negative cases were in the stage pTa.

In our study, low ki-67 immunoexpression was noted in 8(17.39%) cases and high expression in 38(82.6%) cases. Low grade tumors showed low expression in 6 cases(75.0%; **Figure 3D**) and high grade tumors exhibited high expression in 36(94.7%) cases(**Figure 2 D**). Four (5.3%) cases of high grade bladder cancer showed low ki-67 immunoexpression and 2 (25.0%) cases of low grade bladder cancer showed high ki-67 immunoexpression.

As shown in **Figure 5**, all 27 cases of muscle invasive tumor(pT2) revealed high ki-67 expression whereas 6 patients with pTa stage showed low expression.In pT1 staged carcinoma, low and high expression was noticed in 2 and 9 cases respectively.

As seen in **Table 3**, summary of expression of Ki-67 and p-53 was evaluated based on grade and stage of tumour.**% of cells positive for p53**

DISCUSSION

Present study included 46 patients of urothelial carcinoma, and described their demographic details, histopathology and immunohistochemical examination results including histological grading, staging and its' correlation with expression of p53 and ki-67.

p53 nuclear overexpression and strong proliferative Ki-67 index has been previously described to play a role in tumor progression in addition their role as prognostic biomarkers independent of clinical and pathologic parameters is also speculated. Similar to previous studies, our study also showed that maximum number of cases of carcinoma bladder present in the 4th to 6th decade (30 cases, 65.21%), followed by 7th to 8th decade (14 cases, 30.43%).

None of the patients presented uptil the 3rd decade, with only one patient in the 4th decade. Majority of male patients (26 cases) were in between the 4th to 6th decade, *however in females, highest number (10) of cases were observed in 7th to 8th decade of life.*

The M:F ratio in our study was 2.8:1 as compared to the existing literature, where there has been a significant male preponderance, ratio varying from 5:1 to 8.3: 1.

The most common clinical presentation in our study was gross or microscopic hematuria(92%) This was very similar to results by Thakur et al. who had reported hematuria as the sole clinical manifestation in 90.9% of their cases.

Urothelial carcinoma was the only histological type observed in our study of 46 cases, irrespective of the grade and stage of the tumor. This was very similar to results of Popov et al and Senturk et al who had studied 114 and 84 cases respectively. Thakur et al had studied 110 cases of bladder cancer and the most common histological type was transitional carcinoma (urothelial carcinoma; 97.3%), followed by squamous cell carcinoma (2 cases) and adenocarcinoma (1 case).

As tabulated in **Table 4,** the ratio of high grade to low grade tumors showed disparity between various studies, varying from 0.8 to 4.75: 1.0. *Our study demonstrated higher number of high grade and higher stage (stage p T 2)cases as compared to published literature which had shown a ratio of 0.8-1.2: 1.0 as compared to our study which showed a ratio of 4.75:1.0.* This might be attributed to late reference of our patients to the tertiary care medical centre.

Our study showed that 91.3% of the cases overexpressed p53 and only 4 cases(8.7%) were negative for p53. These negative cases were low grade and were pTa on staging. However, one low grade and single pTa tumour showed high expresion (>20%) of p53. *This difference of p53 expression in low grade and high-grade tumors was statistically significant (P=0.00001).*

As shown in **Figure 4,** all the non muscle invasive bladder cancers (p T1) expresed p 53, and the ratio of cases expressing < 20 and >20% of p53 in these p T1 cases was almost same (0.83:1.0). In addition the p T2 cases also showed a ratio of 2:1, when 20% expression of p 53 was taken as a cut off. *This could indicate that it is not the percentage of expression of p53 but the expression itself of p 53 that imposes the patient to a relatively higher risk and thus signifies a higher stage and grade (p T1 and p T2).* Similar results have been reported in literature, where p 53 expression has been reported in 66-100% cases of carcinoma bladder (15, 16, 19, 20), with higher p 53 not only as an indicator of tumor progression, but also an indicator of possiblity of failure of local therapy and thus might suggest a need of early surgical intervention (20).

Our study showed expression of Ki-67 in all the cases of bladder cancer, similar to results by Thakur et al, with low expresion (<20% Ki-67 index) in 75% of low grade cases and high expression in 36/38 high grade cases. Similarly 75% of low stage (p Ta cases) showed low expression and all the muscle invasive cases (p T2) showed high expression (**Figure 5).** *Non muscle invasive cancers (p T1) showed high expression in 9/11 cases (81%), unlike results of Thakur et al who reported high Ki-67 in only 25/58 cases (43.1%; p T1).* Thus, our results of Ki-67 also indicated high expression of Ki-67 in p T1 and p T2 tumors.

CONCLUSION:

Our study demonstrated significant correlation between expression of p 53 and ki-67 with disease stage and grade. However, as compared to p53, Ki-67 demonstrated its' better utility as a better marker to predict severity with high expression in all p T2 tumours and low expression in almost all p T1 tumors. Further, p53 along with Ki-67 are not the stand alone prognostic biomarkers and role of novel biomarkers needs further evaluation.

What Is Already Known On This Topic

1. Urothelial carcinoma are more common in males and commonly occurs in 5th to 6th decade of life.
2. Expression of p 53 and furthermore its higher expression might suggest tumour progression and possibility of failure of local therapy.

What This Study Adds

1. Although our study demonstrated male preponderance, however in our study population, females were more comonly affected as compared to studies from european and western countries.
2. Our study population demonstrated higher number of high grade and higher stage (stage p T 2)cases as compared to literature from western and european countries, this might be attributed to late reference of our patients to the tertiary care medical centre.
3. Our study demonstrated p53 and ki-67 as prognostic biomarkers, in addition to grade and stage of urothelial carcinomas' specifically in a setting of a developing country.

Competing Interests

The authors declare no competing interest.

Acknowledgements:

Authors wish to thank histotechnologists of the Department of Pathology for their technical support.

Table 1: Distribution of cases according to age group and gender.

Age Group (years)	Male	Female
21-40	1(2.17%)	0
41-60	28(60.86%)	2(4.34%)
61-80	4(8.69%)	10(21.7%)
>80	1(2.17%)	0

Table 2: Distribution of cases according to stage and gender.

Stage	Male	Female
p Ta	6(13.04%)	2(4.34%)
p T1	7(15.21%)	4(8.69%)
p T2	21(45.65%)	6(13.04%)

Table 3: Correlation of p53 and ki-67 immunomarker in relation to grade and stage of urothelial carcinoma

	P53			Ki-67	
	Negative	<20%	>20%	<20%	>20%
GRADE					
Low-grade	4	3	1	6	2
High-grade	0	15	23	2	36
STAGE					
pTa	4	3	1	6	2
pT1	0	6	5	2	9
pT2	0	9	18	0	27

Table 4: Comparison of Published literature based on tumor grade and stage.

	Low grade N	pTa Low stage N(%)	High grade N	pT1Higher stage N(%)	pT 2 Higher stage N(%)	Ratio HG :LG
Nilay senturk et al[18]	38	15(13.15%)	46	32(38.09%)	22(26.19%)	1.21:1.0
Brijesh Thakur et al[15]	61	23(20.90%)	49	58(52.72%)	29(26.36%)	0.80:1.0
Present study	8	8(17.39%)	38	11(23.91%)	27(58.69%)	4.75:1.0

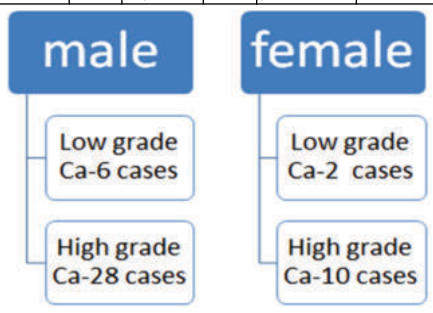

Figure 1 : Distribution of cases according to grade and gender.

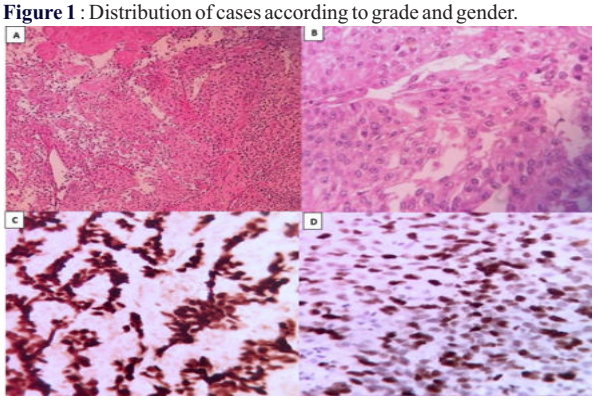

Figure 2: Photomicrograph shows high grade carcinoma at low

magnification (A) and at high magnification (B, Heamatoxylin& eosin stain). P53 & Ki-67 high expression noted respectively(C, D).

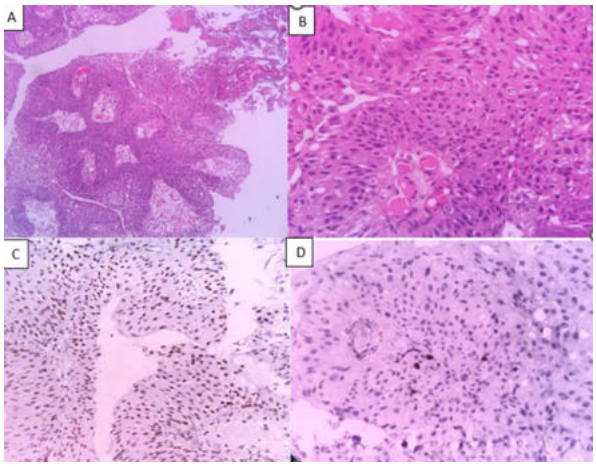

Figure 3: Low grade carcinoma noted at low magnification (A) and high magnification (B). High expression of P 53 and low expression of Ki-67 noted in this low grade tumour ©, D)

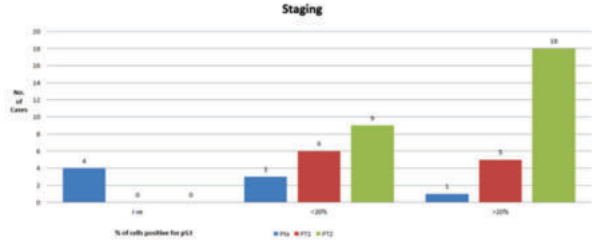

Figure 4: p53 expression levels in different stages of urothelial cancer.

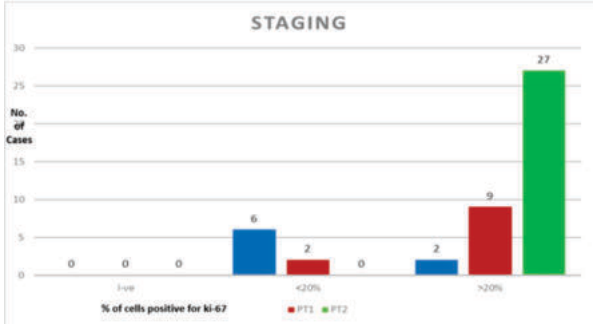

Figure 5: Ki-67 expression levels in different stages of urothelial cancer.

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