



Original Research Article

Isolation of low grade urinary bladder tumors carrying bad prognosis using KI-67 labeling index

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ABSTRACT

Introduction: Even among low grade bladder tumors, there are some which have been found to carry bad prognosis for progression, recurrence and survival. Accurately pinpointing these cases at the time of diagnosis just with the help of routine histopathology is problematic. Ki-67 immunostaining may potentially be of help in identifying such cases, making administration of additional treatment modalities like chemotherapy and radiotherapy possible. There is scarcity of such studies in our country. The present study aims to correlate Ki-67 labeling index with the histopathological type and grade, pathological stage and mitotic index of urinary bladder tumors.

Objective: Objective of the study is to consider the possibility of using Ki-67 labeling index to identify those low grade urinary bladder tumors which carry bad prognosis.

Materials and Methods: All trans-urethral resection of bladder tumor (TURBT) specimens in our institute were analyzed for their histopathological grade, pathological stage, mitotic index and Ki-67 labeling index in the period extending from August 2015 to December 2016. The clinical data were analyzed retrospectively and statistically. SPSS Version 23 (SPSS Inc., Chicago, IL, USA) software was utilized for all statistical analyses. Chi-square analysis was utilized for comparison of groups. Results with $P < 0.05$ were taken as statistically significant.

Results: Ki-67 expression in Low grade urothelial carcinoma (LGUC) showed variation from 1.4% to 41%. The papillary urothelial neoplasm of low malignant potential (PUNLMP) cases showed variation from 16-17%. And the only case of transitional cell papilloma in the study was found to have expression of 9%.

Conclusion: Cases of high (more than 20%) Ki-67 MIB-1 labeling index can be segregated even from among those cases with low histopathological grade, low mitotic index and pTa histopathological stage. Such cases are candidates for multimodality treatment including radiotherapy, chemotherapy etc.

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1. Introduction

According to statistics from the Indian Cancer Registry, bladder cancer is the ninth most common cancer recorded in India, contributing 3.9% of all cancer cases, regardless of gender.¹ The histopathological grade of bladder tumors is usually critical in determining the clinical management and prognosis of patients. However, it has been seen that

even among low grade bladder tumors, there are some which carry bad prognosis for progression, recurrence and survival. Segregating these cases with the help of routine histopathology is problematic. However, identification of such cases can be made easier with the help of Ki-67 immunostaining. Besides, Ki-67 labeling index also proves helpful in assessment of urothelial tumors where a controversy exists in labeling a tumor low grade or high grade by other methods.²

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There is a dearth of literature in our country on the utility of Ki-67 immunostaining in urinary bladder tumors. The present study has tried to fulfill this lacuna.

2. Materials and Methods

The present study is a retrospective institutional based study of paraffin embedded tissue of 80 cases of bladder tumors that came for treatment in our institution from August 2015 to December 2016. The specimens were received in the form of trans-urethral resection of bladder tumor (TURBT) chips. Specimens were fixed in 10% buffered neutral formalin for a period of 24 hours and subsequently the entire specimen was submitted for processing.

Four micron thick sections were made from paraffin blocks and:-

1. Stained with Haematoxylin and Eosin for the assessment of histopathological type and grade, pathological stage, and mitotic index (calculated as number of mitotic figures per 10 high power fields).
2. Immunohistochemical analysis done using MIB-1 antibody provided by Biogenex©, US; following the instructions as provided in the kit.

Ki-67 immunoquantitation was performed utilizing light microscopy at x1000 magnification. Ki-67 labeling index (percentage of tumor cells displaying nuclear staining) was ascertained by counting at least 1000 tumor cells in 10 selected fields displaying the highest immunoreactivity.

The histopathological grade, pathological stage and mitotic index were subsequently correlated with K-67 labeling index, cut value for which was taken as >20%. SPSS Version 23 (SPSS Inc., Chicago, IL, USA) software was utilized for all statistical analyses. Chi-square analysis was used for comparison of groups. Results with $P < 0.05$ were accepted as statistically significant.

3. Results

A total of 80 cases of bladder tumor were subjected to analysis. Maximum number of cases i.e. 26 (32.5%) were found in 61-70 years of age range, 19 cases (23.75%) were observed in 51-60 years followed by 13 cases (16.25%) in 71-80 years, 11 cases (13.75%) were observed in 41-50 years, 7 cases (8.75%) were found in 81-90 years, 2 cases (2.5%) were seen in 21-30 years age, & 2 cases (2.5%) were seen in 31-40 years age group.

Out of 80 cases studied, 70 (87.5%) were males, while 10 (12.5%) were females. Male: female ratio was 7:1. Most common presenting symptom was painless haematuria observed in 63 cases (80%) followed by difficulty in passage of urine in 15 cases (18.75%) and having increased frequency and urgency of micturition in 2 cases (2.5%).

Out of 80 cases, 37 (46.25%) were found to be of Low grade papillary urothelial carcinoma (LGUC), 32 (40%)

were of High grade Papillary urothelial carcinoma (HGUC), 5 (6.25%) were of Adenocarcinoma, 2 (2.5%) were of Papillary Urothelial neoplasm of low malignant potential (PUNLMP), 2 (2.5%) of Spindle Cell Sarcoma, 1 (1.25%) of Nested variant of urothelial carcinoma and 1 case (1.25%) was found to be of Transitional Cell Papilloma (T.C.Papilloma).

Out of 32 cases of high grade urothelial carcinoma, 24 (75%) cases were found to be of High grade papillary urothelial carcinoma, 6 cases (19%) were of high grade urothelial carcinoma with squamous differentiation, while 1 case (3%) was of high grade urothelial carcinoma with glandular differentiation and 1 case (3%) of High grade nonpapillary urothelial carcinoma (Nested variant).

With the exception of 1 case of transitional cell papilloma and 2 cases of papillary urothelial neoplasm of low malignant potential, invasion was studied in the remaining 77 cases. Out of 77 cases, 22 cases (28.6%) were of low grade papillary urothelial carcinoma in which no invasion was observed, 10 cases (13%) were of Low grade papillary urothelial carcinoma with only lamina propria invasion. Five cases (6.5%) were of Low grade papillary urothelial carcinoma which demonstrated muscularis propria invasion. Nine cases (11.7%) were of High grade papillary urothelial carcinoma in which no invasion was seen, two cases (2.6%) which demonstrated only lamina propria invasion, while 21 cases (27.2%) were of High grade papillary urothelial carcinoma which demonstrated muscularis propria invasion.

This study had no cases in pT3 and pT4 category as all cases were sourced through trans-urethral resection.

As can be seen in Table 1, the value of Pearson correlation coefficient was observed to be +0.725 which indicates strong positive correlation between histopathological grade and Ki-67 labeling index and the P value = 0.000, which demonstrates that the results are statistically significant.

Figures 1 and 2 show examples of low and high Ki-67 nuclear positivity respectively.

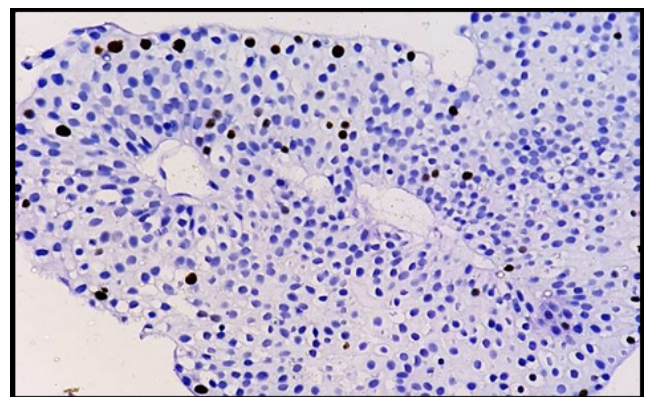
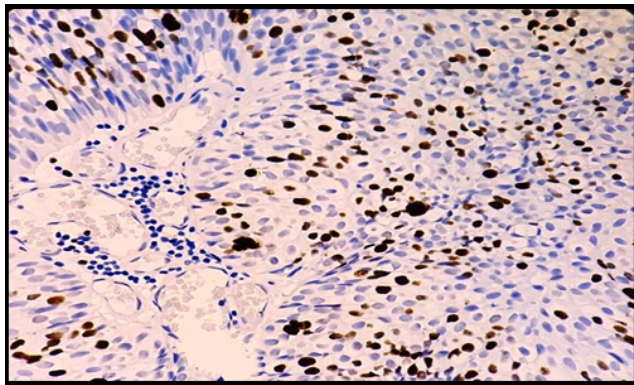


Fig. 1: Ki-67 nuclear positivity <20% in low grade urothelial carcinoma (40 X)

Table 1: Correlation of Ki-67 labeling index with histological grade

Grade	No. of cases with Ki-67 labelling index <20%	No. of cases with Ki-67 labelling index >20%	P value	Pearson correlation coefficient
Low grade and benign (which includes all cases of transitional cell papilloma, PUNLMP, and low grade papillary urothelial carcinoma)	35	5	0.000	+0.475
High grade (including all cases of high grade urothelial carcinoma, adenocarcinoma, and spindle cell sarcoma)	6	34		

**Fig. 2:** Ki-67 nuclear positivity >20% in High grade non-invasive papillary urothelial carcinoma (40 X)

Also as demonstrated in Table 1, six of the total 41 low grade bladder tumor cases in this study had Ki-67 labeling index above 20%. On the other hand, five out of 39 high grade cases had a Ki-67 labeling index below 20%.

As can be seen in Table 2, the value of Pearson correlation coefficient was observed to be =0.423 which indicates moderate correlation between pathological stage and Ki-67 labeling index, and the P value = 0.000, which demonstrates that the results are statistically significant.

Also, among the non-invasive (pTa) tumors, 10 out of total 34 such cases had above 20% Ki-67 labeling index. It was found that, even though most of such tumors were of high grade, two low grade tumors with pTa stage also had a high Ki-67 labeling index. Similarly three low grade tumors with pT1 stage and one low grade tumor with pT2 stage, also had a high Ki-67 labeling index.

On the contrary, as many as 8 out of total 33 cases in pT2 stage were found to have a Ki-67 labeling index of less than 20%. Thus, the correlation of Ki-67 index with pathological stage is less than its correlation with histopathological grade (as noted IN Table 2), and with mitotic index (as discussed in Table 3).

As can be seen in Table 3, the value of Pearson correlation coefficient was observed to be +0.759, which

indicates strong positive correlation between mitotic index and Ki-67 labeling index, and the P value = 0.000, which demonstrates that the results are statistically significant.

Table 3 also demonstrates that none of the cases with mitotic index of more than 20/10 hpf had a Ki-67 labeling index below 20%. But there were occasional (n=3) cases with low mitotic index (<10/10 hpf), that had a Ki-67 labeling index above 20%.

4. Discussion

Ki-67 is a nuclear protein which is present during G1, S, G2 and M phases of continuously cycling cells, though it is not present in G0 cells. Though not well characterized, the genetic locus of Ki-67 has been assigned to chromosome 10. It has been shown by several studies that cell proliferative activity as defined by Ki-67 labeling index correlates with the tumor growth fraction. MIB-1 monoclonal antibody detects a formalin-resistant epitope of Ki-67 antigen thereby making it suitable for use on routinely processed paraffin-embedded tissue.³

Different Ki-67 equivalent antibodies recognize different epitopes of Ki-67 antigen and, therefore, have dissimilar sensitivity and specificity. While comparing different available antibodies, Lindboe et al observed that MIB-1 antibody had a higher sensitivity than other antibodies, and it also gave the best visual staining, with more diffusely and strongly stained nuclei.⁴ The prototypic Ki-67 antibody had the limitation of being usable only on frozen section specimens. Moreover, it was observed that staining with MIB-1 led to demonstration of considerably higher labeling indices as compared to the prototypic Ki-67 antibody, probably due to better preservation of epitopes after formaldehyde fixation and antigen retrieval than by freezing the tissue.^{5,6}

The labeling index can be defined as the percentage of immunoreactive nuclei.⁴ MIB-1 Ki-67 equivalent antibody was used in present study too to determine the labeling index.

In present study, Ki-67 MIB-1 labeling index was observed to be more than 20% in 14.6% of all the low grade

Table 2: Correlation of Ki-67 MIB 1 labeling index with pathological stage

Stage	No. of cases with Ki-67 labeling index less than 20%	No. of cases with Ki-67 labeling index more than 20%	P Value	Pearson correlation coefficient
pTa	24	10	< 0.001	+ 0.423
pT1	8	5		
pT2	8	25		

Table 3: Correlation of Ki-67 labeling index with mitotic index (No. of mitotic figures/ 10 hpf)

Mitotic Index	No. of cases with MIB1 labeling index below 20%	No. of cases with Ki-67 labeling index above 20%	P Value	Pearson correlation coefficient
0-10/10hpf	39	3	<0.001	+ 0.759
11-20/10hpf	1	26		
21-30/10hpf	0	9		
31-40/10hpf	0	1		
41-50/10hpf	0	1		

bladder tumors whereas 87.2% of all the high grade tumors had more than 20% labeling index. Such tumors merit adjuvant treatment utilizing radiotherapy and chemotherapy. Margulis V et al did Kaplan – Meir analyses of their data on urinary bladder cancer cases and observed that the Ki-67 cut off of 20% reactivity was the best discriminator for both bladder cancer recurrence and survival.⁷ 44.1% of their low-grade tumors and 49.07% of high grade tumors demonstrated labeling index equal to or more than 20%. Gontero and colleagues have observed Ki-67 labeling index more than 20% as predictor of recurrence in superficial low grade bladder cancer cases.⁸

Other researchers have either not mentioned the cut off value in their studies or used a different cut off value. Santos et al, using more than 18% immunoreactivity as their cut off value observed that, in Grade 1, 29.2% patients came in this group and in Grade 2, 70.8% patients had more than 18% immunoreactivity.⁹ Ding W et al, using Ki-67 immunoreactivity equal to or more than 25% as their criteria of Ki-67 positivity observed that 32.5% of cases with non-muscle invasive bladder cancer were Ki-67 positive.¹⁰ Tsuji M et al observed in their study that MIB-1 labeling index averaged 17.8 in Grade 2 and 32.3 in Grade 3 patients.¹¹

Goyal S et al observed Ki-67 index $11.8 \pm 8.23\%$ in low grade and $35.86 \pm 17.55\%$ in high grade papillary urothelial bladder cancer. These researchers also found that cut-off values for Ki-67 and mitotic indices can confirm high grade bladder carcinoma in equivocal cases. They also observed that Ki-67 and mitotic count can serve as potential and reliable indicators of muscle invasion. Thus they reported that Ki-67 equal to or more than 32.5% and 14 or more mitoses per 10 high power fields was 100% specific for high grade bladder carcinoma. Besides, Ki-67 equal to or more than 59% and 37 or more mitoses per 10 high power fields were 100% specific for muscle invasive bladder carcinoma.¹² In present study, also, Ki-67 MIB-1

labeling index values correlated well with mitotic indices and pathological staging of the tumor.

The Ki-67 labeling index value for low and high grade tumors by various researchers has been shown in tabular form in Table 4.

In another table, Table 5, comparison of Ki-67 labeling index values of present study with those of Margulis V et al⁷ has been done according to age group, gender, tumor stage and tumor grade. While the age and sex distribution in both the studies is comparable, present study had comparatively higher percentage of patients in positive cohort in pT2 stage. Present study also had a higher percentage of Ki-67 labeling index positivity among the patients with high grade tumors.

In Table 6, a comparison of range of Ki-67 labeling index values for low grade and high grade tumors of present study with two other recent studies is shown.

Using Ki-67 expression, Gonul IT et al could distinguish “papillary urothelial neoplasm of low malignant potential” from other grade 1 urothelial tumors, thus obtaining more precise prognostic information.¹⁴ There were two such cases in present study.

Only 33 out of 80 cases were non-invasive in present study whereas approximately 80% of bladder tumors have been found to be non-invasive in literature.^{9,15} The underlying reason maybe that our patients present late to the hospitals for treatment. Any difference in biological behavior needs to be investigated.

Most of the patients in present study were males. This is in accordance with other reports in literature.^{7,15}

For superficial bladder tumors, classical histopathological markers do not define risk profiles and, consequently, there are no optimal treatment approaches and follow-up schedules. The course of disease of such tumors is, therefore, uncertain.¹⁶ By adding Ki-67 index to the risk model, it became possible to ascertain subset of

Table 4: Comparison of Ki-67 Labeling Index value with other studies

Study	Year	Geographical Area	Ki-67 cut off value used	Low grade urothelial carcinoma	High grade urothelial carcinoma
Present study	2017	India	>20%	14.6%	87.2%
Ding W et al	2014	China	>25%	32.5%	NA
Margulis V et al	2009	USA	>20%	44.1%	49.07%
Santos L et al	2003	Portugal	>18%	29.2%	70.8%
Tsuji N et al	1997	Japan	>25%	NA	50%

NA – Not available

Table 5: Comparison of Ki-67 Labeling Index values in accordance with various age groups, gender, tumor stage and grade with another study

Characteristic	Entire cohort (No. of patients)		Ki-67 positive cohort (No. of patients)		Ki-67 negative cohort (No. of patients)	
	Present study	Margulis V et al	Present study	Margulis V et al	Present study	Margulis V et al
Mean age (y)	62	67.5	64.9	67.9	60.2	67.2
Gender						
Male	70	570	35	281	35	289
Female	10	143	5	63	5	80
Pathological stage						
pTa	34	NA	10	NA	24	NA
pT1	13	89	5	32	8	57
pT2	33	160	25	72	8	88
pT3	NA	335	NA	167	NA	168
pT4	NA	129	NA	73	NA	56
Tumor Grade						
Low	41	120	6	53	34	57
High	39	593	35	291	5	302

NA-Not available

Table 6: Comparison of range of Ki-67 labeling index values of low grade and high grade bladder tumors with other studies

Study	Year	Geographical area	Range of Ki-67 labeling index for low grade tumors	Range of Ki-67 labeling index for high grade tumors
Present study	2017	India	1.4 – 41	8 – 70
Sangwan M et al ¹³	2015	India	2 – 38	46 – 90
Goyal S et al ¹²	2014	India	3.65 – 20.11	18.31 – 53.41

low grade urothelial superficial papillary carcinomas with a worse course of disease, which can be closely followed up and may benefit from adjuvant treatments.⁹ Three out of total 41 low grade tumors in present study fall in this group.

Radical cystectomy is the standard treatment for patients who have refractory non-muscle invasive and muscle invasive urothelial carcinoma of the urinary bladder.^{17,18} Standard prognostic features like pathological stage and grade are of limited use in prediction of outcomes in these patients. Molecular biomarkers like Ki-67 may play a role in stratifying the heterogeneous patient population undergoing radical cystectomy into risk categories which can help in determining as to whether patients should be treated by observation or with adjuvant therapy.¹⁹ Recently, Tanabe K et al have commented that muscle invasive bladder

cancer patients with high Ki-67 expression status might derive benefit from chemoradiotherapy based multimodal approaches in terms of prognosis and quality of life as a result of bladder preservation.²⁰

Even though follow-up of the patients was not a part of present study, several studies have demonstrated an association between proliferation and tumor grade, stage, recurrence and prognosis.^{21,22} Quintero A et al concluded that tumor proliferation measured by Ki-67 labeling index is related to tumor recurrence, stage progression, and is an independent predictor of disease free survival, progression free survival and cancer-specific overall-survival in TaT1 bladder urothelial cell carcinoma.¹⁵

Interestingly, a recent systematic review and meta-analysis has stated that significant correlations between

high Ki-67 expression and survival outcome (recurrence-free/ progression-free/ overall cancer-specific survival) are present only in European – American patients and it does not reach significant level in Asian population.²³ Thus, further research in this regard in Asian countries is called for.

Some researchers have linked high Ki-67 expression with distant metastasis in Cancer. They have commented that elevated MIB 1 labeling index may induce epithelial-to-mesenchymal transition by increasing the expression of vimentin, which enhances cancer-cell invasion and metastases.²⁴ Information about metastases in patients in present study is not available.

There were some limitations in present study. Being retrospective in nature, no follow-up about patients studied is available. Being a single observer study, the present study did not evaluate inter-observer variability in determining results. Besides, the present study is a single-institution study.

5. Conclusion

Cases of high (more than 20%) Ki-67 MIB-1 labeling index can be segregated even from among those cases with low histopathological grade, low mitotic index and pTa histopathological stage. Such cases are candidates for multimodality treatment including radiotherapy, chemotherapy etc.

For segregating tumors with better and worse prognosis, it is needed to adopt uniform cut-off percentage of Ki-67 MIB-1 labeling index in all international studies to enable logical comparison of their results.

As a recent systematic review and meta-analysis²³ has stated that significant correlation between high Ki-67 expression and survival outcome are present only in European-American patients, and it does not reach significant level in Asian population, there is need for multi-institutional follow-up studies in our country to find the exact value of determination of Ki-67 expression.

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Self only for purchasing the antibody to Ki-67 antigen for immunohistochemical analysis.

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