Correlation of Mitotic Indices, AgNor Count, Ki-67 and Bcl-2 with Grade and Stage in Papillary Urothelial Bladder Cancer

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Purpose: To evaluate the mutual inter-relationship of mitotic indices, argyrophilic nuclear organizer regions (AgNOR) count, Ki-67 and B-cell lymphoma 2 protein (bcl-2) in papillary urothelial bladder cancer (pUBC), and their correlation with grade and stage. To establish the cut-off values of these markers to detect high grade and muscle invasive bladder cancer.

Materials and Methods: Fifty-four patients with primary pUBC who underwent transurethral resection / radical cystectomy were analyzed retrospectively. Cell proliferation was assessed by Ki-67 labelling index, mean AgNOR count, mitotic count, mitotic activity index and mitosis/volume index. Immunohistochemistry was done to see bcl-2 and Ki-67 expression. Correlation of these indices with tumor grade and stage and amongst themselves was assessed. The receiver operating characteristic (ROC) curves were drawn to establish the cut-off values.

Results: We found a strong positive correlation of mitotic indices and Ki-67 with tumor grade ($P = .000$), stage ($P < .05$) and bcl-2 ($P = .000$). AgNOR count correlated positively with the grade ($P = .006$), mitotic indices and Ki-67 ($P = .032$) but not with tumor stage and bcl-2. Cytoplasmic bcl-2 immunopositivity was seen in 42.3% of low grade pUBC and 85.7% of high grade pUBC cases ($P = .001$). bcl-2 positivity was seen in 85% of muscle invasive pUBC as compared to only 52.9% of superficial cases. Ki-67 $\geq$ 32.5%, $\geq$ 14 mitoses/10 high power fields (hpf), $\geq$ 11.20 mitoses/mm^2, $\geq$ 0.75 mitoses/100 tumor cells and AgNOR $\geq$ 11.55 are 100% specific for high grade bladder carcinoma. Ki-67 $\geq$ 59% and mitoses $\geq$ 36.50 per 10 hpf can indicate muscle invasion with 100% specificity.

Conclusion: Cut-off values for Ki-67, mitotic indices and AgNOR can confirm high grade bladder carcinoma in equivocal cases. Ki-67 and mitotic count can serve as potential and reliable indicators of muscle invasion.

Keywords: Ki-67 antigen; mitotic index; predictive value of tests; urinary bladder neoplasms; diagnosis.

INTRODUCTION

Bladder cancer is the fourth most common cancer in men and ninth most common in women worldwide, leading to significant morbidity and mortality which poses a major economic burden on global health care systems.[1] Bladder cancer is one of the costliest diseases to treat due to long term survival associated with non-muscle-invasive disease.[2] Conventional parameters like tumor size, grade and stage alone have limited role in specifying the risk of progression, recurrence or response to treatment for an individual patient, as bladder cancer patients with the same grade and stage often show markedly different clinical outcome.[3] Despite their imperfections presently, there are no prognostic markers for bladder cancer which are superior to conventional grading and staging.[4] Overall 5 year survival drops to 38.5% for stage T2 as compared to 70% in stage T0 and T1 bladder cancer patients.[5] Newer reproducible markers which can detect muscle invasion and are confirmatory for high grade bladder cancer may serve as a better guide for patient management and clinical outcome, than conventional grade and stage alone..

Evolution of bladder cancer comprises a multistep process involving various alterations in the activity of genes regulating the cell division and apoptosis. B-cell lymphoma 2 protein (bcl-2) family, a group of closely related proteins and several other genes including c-myc, H ras, ABL, Apo-1, and p53 play a major regulatory role in apoptosis. bcl-2 overexpression has been associated with reduced tumor sensitivity to chemotherapy and radiotherapy.[6] bcl-2 expression in different malignancies is variable and depends on cell lineage.[7] bcl-2 overexpression is a poor prognostic factor in high grade lymphomas, leukemia, neuroblastoma and prostatic cancer, while lung and breast cancer patients with bcl-2 expression have a better chance of survival.[8] Overexpression of bcl-2 is therefore, of potential relevance to the pathogenesis and progression of bladder cancer and its response to therapeutic interventions.

Proliferative activity is currently being evaluated as an indicator of biological aggressiveness in bladder cancer. Mitotic count is apparently the most convenient marker to assess the proliferative status of a tumor. Mitotic indices especially mitosis/volume (M/V index) has been shown to be an independent prognostic indicator of survival in papillary urothelial bladder cancer (pUBC), although most of the studies regarding this largely come from one group of researchers.[9-11]

Nuclear organizer regions (NOR) are loops of ribosomal DNA (rDNA) in cell nucleoli which are visible in interphase on silver staining.[12] Argyrophilic NORs (AgNORs) are nonhistone proteins associated with the synthetic activity localized to the NORs and provide an estimate of cellular proliferation.[13] In bladder cancer, correlation between AgNORs and the tumor behavior remains still disputatious.[14,15]

Tumor growth fraction can be assessed by using a monoclonal antibody Ki-67 reacting with a nuclear antigen, which is expressed in the S, G2 and M phases of the cell cycle. Proportion of Ki-67 labelled cells in a given cell population (Ki-67 Labelling Index) has been found to be related to bladder tumor recurrence, grade and stage in various studies.[16] However, absolute cutoff values to confirm high grade and invasive pUBC have not been reported yet.

To the best of our knowledge, very few studies in literature have studied the diagnostic role of these three proliferative markers and bcl-2, in combination, and cut-off values to detect high grade and muscle invasive pUBC (which is known to have a poor clinical outcome) have never been proposed in literature before. Current literature is confounding regarding the utility of bcl-2 in bladder cancer. Therefore, the present study was conducted with the aims: 1) to compare and determine the correlation of proliferative markers (mitotic indices, AgNOR count and Ki-67 Labelling Index) and bcl-2 with grade and stage in pUBC: 2) to establish the cut-off values which can reliably distinguish high grade from low grade and, invasive from superficial papillary bladder cancer.

MATERIALS AND METHODS

Subject Population

This was a single-institution retrospective study approved by the institutional ethics committee and the need to obtain informed consent was waived. Fifty four patients (45 males, 9 females) of primary bladder cancer presenting in the urology outpatient department from October 2008 to April 2011, who were treated with trans-urethral resection of bladder tumor (TURBT) or cystectomy and found to have primary histologically confirmed pUBC, were included in the study. Clinical details, radiological findings and adequate tissue material were available in all these cases for tumor grading and staging. Radical cystectomy specimen was available in ten cases; TURBT material was evaluated for the rest of the 44 cases.

The tissue was fixed in 10% buffered formalin, routinely processed and embedded in paraffin blocks. The 5 µm thick sec-

Table 1. Grade and stage wise distribution of cases.

Stage	Low Grade	High Grade	Total, no (%)
pTa	15	3	18 (33.3)
pT1	8	8	16 (29.6)
pT2	1	10	11 (20.4)
pT3	2	5	7 (12.9)
pT4	0	2	2 (3.8)
Total, no (%)	26 (48.1)	28 (51.9)	54 (100)

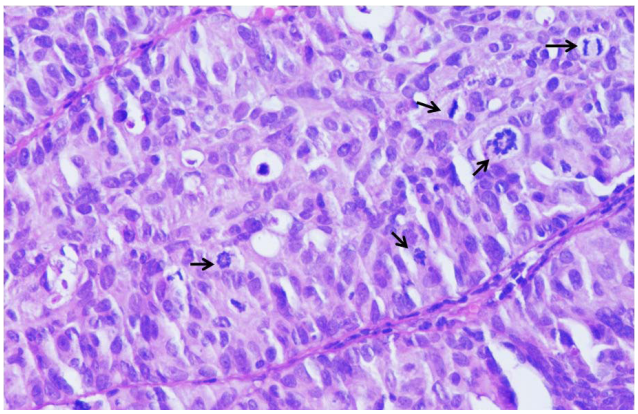

Figure 1. High mitotic count with atypical mitoses in high grade papillary bladder cancer (Hematoxylin and Eosin, ×400).

tions were taken for hematoxylin and eosin and AgNOR staining. Twenty TURBT samples reported as non-neoplastic (inflammatory) in patients suspected to have bladder malignancy, in the study period were taken as controls.

Histological Grading and Staging

Hematoxylin and eosin stained sections were examined and graded in the worst differentiated area. Grading and TNM staging was done according to World Health Organization/International Society of Urological Pathology (WHO/ISUP) 2004 and the American Joint Committee on Cancer (AJCC) classification system.[17,18] Clinico-radiological correlation was done to assign stage in cases of advanced tumors (beyond pT2).

Mitotic Indices

The most cellular tumor areas were selected for mitotic counts, avoiding the necrotic areas, at × 400 magnification. Mitotic counts were counted per 10 high power fields (hpf) on hematoxylin and eosin sections. Mitoses/Volume (M/V) Index is defined as number of mitotic figures per square millimeter of tumor (in Motic microscope, 10 consecutive hpf correspond to 1.25 mm^2). The Mitotic Activity Index (MAI) was calculated as number of mitotic figures per 100 tumor cells counted under × 400 magnification.

AgNOR Staining

For AgNOR staining, working solution was made of two parts of 50% aqueous silver nitrate solution, made in distilled water and one part of 2% gelatin in 1% aqueous formic acid. Deparaffinized sections were incubated at room temperature for half an hour in dark after adding freshly prepared working solution. Slides were washed, air dried and mounted using DPX. Number of AgNORs in 100 randomly selected tumor cells were counted under oil immersion lens. AgNORs in clusters were counted separately wherever possible and the mean AgNOR count/nucleus was calculated.

bcl-2 and Ki-67 Immunoanalysis

Streptavidin-biotinylated immunoperoxidase method was used for demonstration of bcl-2 and Ki-67 antigen. Four µm thick serial sections on poly-L-lysine coated slides were deparaffinized in xylene, rehydrated using graded alcohol and washed with tris buffer (pH 7.4). For antigen retrieval sections were placed in 0.01 M citrate buffer (pH 6.0) for 10 min at 98ºC in EZ Retriever system (v.2.1, Biogenex, Fremont, California, USA). Sections were cooled to room temperature, and washed with tris buffer. Endogenous peroxide was blocked by 4% H_2O_2 for 15 min. Sections were incubated overnight at 4-8ºC with the ready to use primary mouse monoclonal antibody (bcl-2: 226M-98 Cell Marque, Rocklin, California, USA; Ki-67: PM375 AA Biocare Medical, Concord, California, USA). Sections were treated with biotinylated antimouse link antibody, followed by preformed streptavidin conjugated horseradish peroxidise complex for 30min. Diaminobenzidine tetrahydrochloride (DAB) (0.6 mg/mL in Tris buffer saline, pH 7.6 containing 0.04% hydrogen peroxide) was used to develop brown color. Harris hematoxylin was used to counterstain the slides.

For positive controls of bcl-2 and Ki-67 follicular lymphoma and normal tonsil were stained respectively. A negative control (with primary antibody omitted) was taken along with each batch. Immunohistochemical analysis was performed by two pathologists in consensus, blinded to tumor grade and stage. Fraction of positively stained tumor cells was scored semi-quantatively after examining at least 5 hpf (×400) for each case. At least moderate cytoplasmic or perinuclear staining in 10%

Table 2. Correlation of mitotic indices, mean AgNOR count, Ki-67 labelling index and bcl-2 with tumor grade.

Variables	Controls	Low Grade	High Grade	Correlation Coefficient	*P*
Mean mitotic count	0.32 ± 0.45	3.30 ± 2.54	15.30 ± 20.04	0.679	.0001
Mitoses/Volume Index	0.04 ± 0.07	2.64 ± 2.03	12.55 ± 16.3	0.581	.001
Mitotic Activity Index	0.0	0.20 ± 0.07	92. ± 0.96	0.458	.000
Mean AgNOR count	1.7 ± 4.2	1.86 ± 6.39	4.04 ± 8.86	0.312	.006
Ki-67 Labelling Index	4.5 ± 2.68	11.88 ± 8.23	35.86 ± 17.55	0.711	.000
Proportion of bcl-2 positive cases	0.0	42.3%	85.7%	0.454	.001
Mean bcl-2 immunopositivity*	0.0	19.35 ± 23.16	49.57 ± 3.49	0.486	.000

Key: bcl-2, B-cell lymphoma 2 protein; AgNOR, argyrophilic nuclear organizer regions.
*Percentage area positive for bcl-2.

of tumor cells was required to define bcl-2 positivity. Staining intensity of bcl-2 immunopositivity was determined as mild/moderate/high by comparison with the staining of lymphocytes (positive internal control). Mean bcl-2 immunopositivity was scored according to the percentage of tumor area showing bcl-2 staining.

For assessing tumor growth fraction, random fields were selected in well preserved areas in each section. Proportion of tumor cells showing nuclear staining was counted and expressed as percentage (Ki-67 Labelling Index). At least 1000 tumor cells were assessed for each case, unless the section was smaller, in which case all the cells were counted.

Statistical Analysis

The statistical package for the social science (SPSS Inc, Chicago, Illinois, USA) version 17.0 was used for analysis. For comparison between two groups, Student's *t* test was employed and one way analysis of variance (ANOVA) was used in case of more than two groups. Spearman's coefficient of correlation (Rho) was calculated to assess the association of the proliferative indices and bcl-2 with tumor stage and grade. Contingency

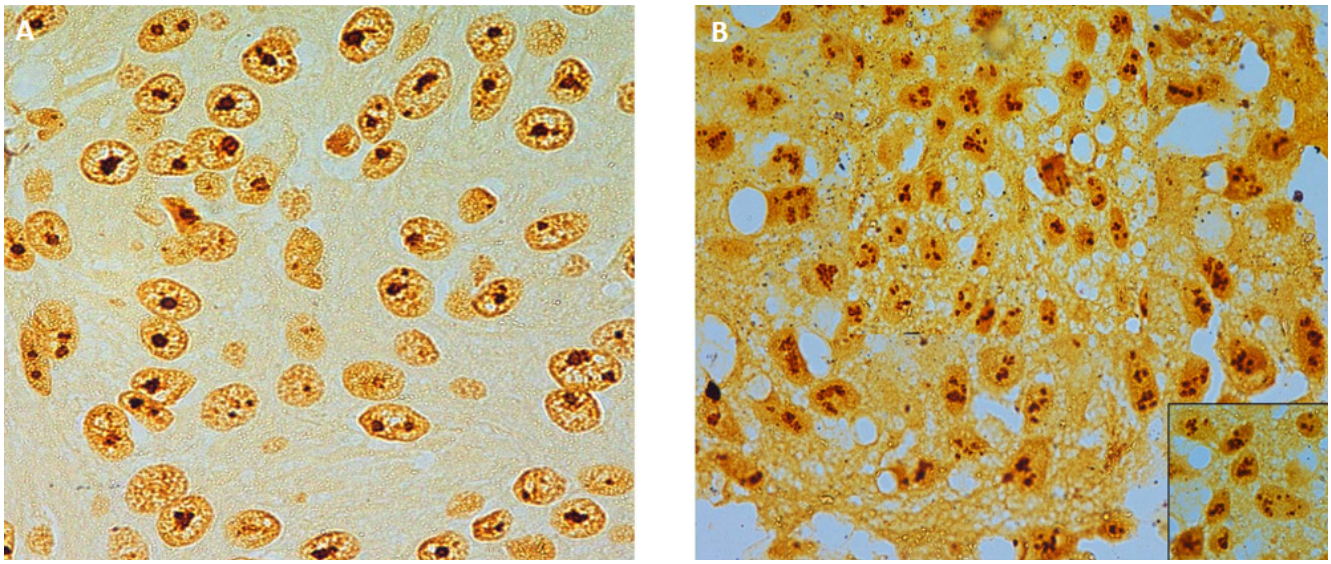

Figure 2. a) Low AgNOR count in low grade papillary bladder cancer (AgNOR, ×1000). b) High AgNOR count in high grade papillary bladder cancer (AgNOR, ×1000).

Table 3. Correlation of mitotic indices, mean AgNOR count, Ki-67 labelling index and bcl-2 with tumor stage.

Stage	pTa	pT1	pT2	pT3	pT4	*P*	Correlation coefficient
Mitotic count	10.43 ± 5.0	11.59 ± 11.56	17.8 ± 24.6	7.28 ± 9.76	15.0 ± 16.9	.004	0.517
Mitoses/Volume Index	9.54 ± 4.36	9.27 ± 9.25	14.2 ± 19.7	5.83 ± 7.80	12.0 ± 13.5	.008	0.509
Mitotic Activity Index	0.56 ± 0.23	0.69 ± 0.50	1.04 ± 1.26	0.23 ± 0.60	0.75 ± 1.06	.009	0.238
Mean AgNOR count	2.44 ± 7.17	3.63 ± 8.51	4.65 ± 8.10	5.66 ± 2.33	10.1 ± 1.4	.285	NS
Ki-67 Labelling Index	13.78 ± 14.16	24.31 ± 14.91	36.1 ± 18.0	29.0 ± 21.2	37.5 ± 31.8	.000	0.490
bcl-2 positive cases, (%)*	5/18 (27.7)	13/16 (81.2)	9/11 (81.8)	6/7 (85.7)	2/2 (100)	.002	0.492
Mean bcl-2 positivity**	11.39 ± 17.18	36.63 ± 28.05	55.9 ± 31.2	55.0 ± 29.7	50.0 ± 49.4	.000	0.580

Key: NS, not significant ; bcl-2, B-cell lymphoma 2 protein; AgNOR, argyrophilic nuclear organizer regions .
*Proportion of bcl-2 positive cases, ** percentage area positive for bcl-2.

tables were drawn and Fischer's exact test or Pearson's Chi square test of proportions were used to assess grade and stage significance for bcl-2 positivity. Pearson's correlation coefficient was used to assess the mutual correlation between the proliferative indices and bcl-2 immunopositivity. All *P* values < .05 were considered statistically significant. Receiver Operating Characteristic (ROC) curves were drawn to determine the cut-off values (with highest average sensitivity and specificity) for distinction between two groups.

RESULTS

Demographic Profile

Mean age of 54 pUBC patients (45 men, 9 women) was 58.4 years, age range 35-95 years. Control group comprised of twenty non-neoplastic cases with mean age of 54.5 years (17 males, 3 females), age range 30-75 years. Distribution of cases according to histological grade and stage is shown in Table 1. Keeping in view the small number of cases in each stage, and considering the fact that invasion into lamina propria is associated with sharp decrease in patient survival, we divided our cases into prognostically significant groups: superficial (pTa and pT1, n = 34) and invasive (stage pT2 and beyond, n = 20).

Mitotic Indices

A significant difference was noted in the mean mitotic count,

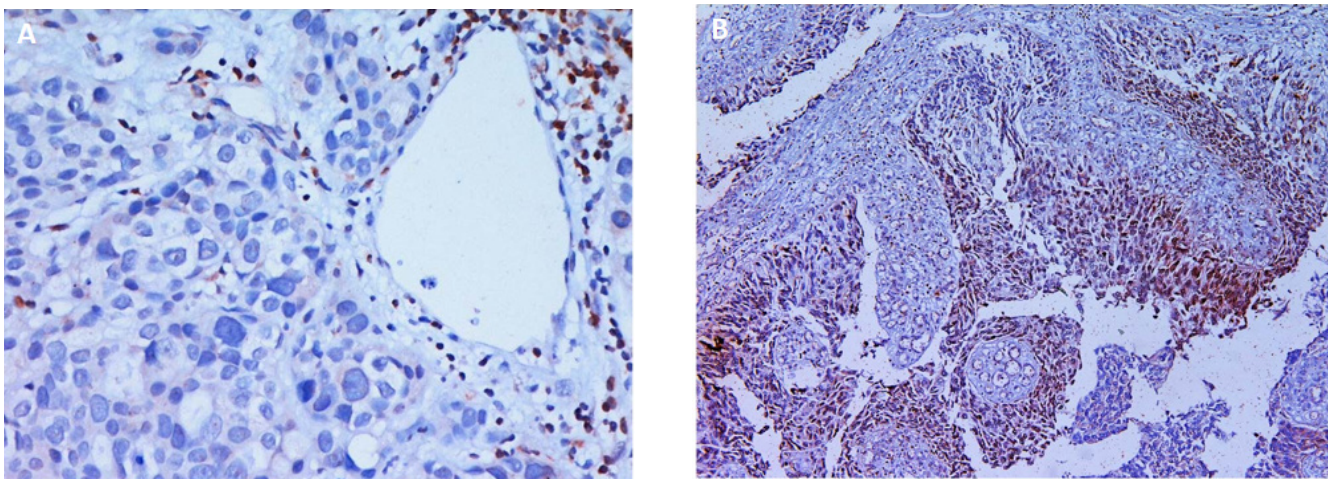

Figure 3. a) Very faint cytoplasmic bcl-2 positivity in low grade papillary bladder cancer with lymphocytes serving as strong internal positive controls (bcl-2 immunostain x 400) b) strong cytoplasmic staining of bcl-2 in high grade papillary bladder cancer (bcl-2 immunostain x 100).

Table 4. Mitotic Indices, mean AgNOR count, Ki-67 labelling index and bcl-2 in superficial (pTa and pT1) and invasive (pT2 and beyond) papillary urothelial bladder cancer.

Variables	Superficial	Invasive	*P*
Mean mitotic count	8.09 ± 11.32	17.60 ± 16.78	.016
Mitoses/Volume Index	6.66 ± 9.56	14.08 ± 13.43	.022
Mitotic Activity Index	0.36 ± 0.63	0.85 ± 0.94	.029
Mean AgNOR count	7.80 ± 3.08	7.44 ± 3.92	.714
Ki-67 Labelling Index	18.74 ± 15.26	33.80 ± 19.49	.003
Proportion of bcl-2 positive cases	52.9%	85.0%	.021
Mean bcl-2 immunopositivity*	23.26±25.94	55.00 ± 30.43	.000

* Percentage area positive for bcl-2.
Key: bcl-2, B-cell lymphoma 2 protein; AgNOR, argyrophilic nuclear organizer regions.

MAI and M/V index of the low and high grade pUBC (Table 2) as well as between the various stages of the tumor (Tables 3 and 4). Spearman's correlation coefficient showed a significant positive correlation of the mitotic indices with the tumor grade and stage. Atypical mitoses including- tri/quadripolar and multipolar mitoses, ring mitoses, asymmetrical mitoses were easily identified in high grade tumors (Figure 1).

AgNOR Counts

The mean AgNOR count in pUBC cases ranged from 3.2-18.8 with a mean of 8.7 ± 3.3 as compared to 4.2 ± 1.7 in controls. The mean AgNOR count was significantly lower in low grade pUBC as compared to high grade tumors (Figure 2), however, the difference between the superficial and muscle invasive groups was not statistically significant and no correlation was observed between mean AgNOR count and tumor stage (Tables 2 and 3).

bcl-2 and Ki-67 Labelling Index

In the control group, cytoplasmic bcl-2 immunopositivity was seen in one to two basal cell layers of transitional epithelium. Stromal lymphocytes were strongly positive for bcl-2, thereby acting as internal controls (Figure 3). In pUBC cases, expression of bcl-2 was seen mainly in superficial and non-basal layers of urothelium. bcl-2 positivity was mainly cytoplasmic (Figure 3). bcl-2 expression was seen in 35/54 (64.8%) cases of pUBC. Not only the proportion of bcl-2 immunopositive cases was significantly higher in high grade group, but also the percentage tumor area positive for bcl-2 was significantly higher in high grade as compared to low grade (Fisher's exact test, $P = .001$) (Table 2).

We observed an increase in the proportion of bcl-2 immunopositive cases with increasing tumor stage and this difference was found to be statistically significant ($P = .002$, Spearman's correlation coefficient, $r = 0.492$). Mean bcl-2 immunopositivity was significantly higher in invasive cases as compared to superficial ones (Table 4). No significant correlation was found between the staining intensity and the tumor grade or stage.

Ki-67 immunostaining showed variable intense nuclear positivity (Figure 4) in all pUBC cases, which was significantly higher than in control group. Mean Ki-67 labelling index (Ki-67 LI) was significantly higher in high grade and invasive pUBC as compared to low grade and superficial cases (Tables 3 and 4). Box and Whisker plots depicting the median and distribution range of bcl-2 and Ki-67 percentage immunopositivity in low vs. high grade and superficial vs. invasive pUBC cases are shown in Figure 5.

Mutual Correlation Between Proliferative Indices and bcl-2 Expression

We found a strong positive correlation of the proliferative markers (mitotic count, Ki-67) with bcl-2 immunopositivity ($P = .000$, $r = 0.596$). However, no correlation was found between mean AgNOR count and bcl-2 expression. Mitotic count and Ki-67 LI showed a significant positive correlation with mean AgNOR count ($P = .032$, $r = 0.292$).

Mean mitotic count of bcl-2 positive cases was 14.97 ± 14.39/10 hpf and of bcl-2 negative cases was 5.42 ± 11.93/10 hpf, and this difference was found to be statistically significant ($P = .017$). Mean Ki-67 LI (29.37 ± 17.23) in bcl-2 positive

Table 5. Receiver operating characteristic analysis for distinction of high grade papillary bladder cancer.

Variables	AUC	Cut-off value	Sensitivity (%)	Specificity (%)	95% CI	*SE*	*P*
Mitotic count	0.890	5.5	75.0	88.5	0.800-0.981	0.046	.000
Mitoses/Volume Index	0.890	2.0	92.9	76.9	0.800-0.981	0.046	.000
Mitotic Activity Index	0.793	0.75	57.1	100.0	0.669-0.916	0.063	.000
Mean AgNOR count	0.680	8.70	53.6	92.3	0.531-0.829	0.076	.023
Ki-67 Labelling Index	0.910	21.5	75.0	84.6	0.837-0.983	0.037	.000
Mean bcl-2 positivity	0.780	32.5	64.3	84.6	0.656-0.905	0.064	.000

Key: CI, confidence interval; SE, standard error; AUC, area under the curve; bcl-2, B-cell lymphoma 2 protein; AgNOR, argyrophilic nuclear organizer regions.

cases was significantly higher than in bcl-2 negative cases (15 ± 16.86), $P = .005$. So bcl-2 immunopositivity was associated with higher mitotic count and Ki-67 LI.

ROC Analysis

ROC curves were drawn to calculate area under the curve (Figure 6) and cut-off values with highest average sensitivity and specificity for all the parameters to distinguish high grade from low grade (Table 5). In addition to cut-off value shown in the table, ROC analysis also showed Ki-67 LI ≥ 32.5%, mitotic count ≥ 14/10 hpf, M/V index ≥ 11.20 mitoses/mm^2, MAI ≥ 0.75 and AgNOR count ≥ 11.55 were found exclusively in high grade pUBC (specificity = 100%). None of the high grade tumors had Ki-67 LI < 9%. Similar ROC analysis for tumor stage revealed, 100% specificity of Ki-67 ≥ 59% and mitotic count ≥ 36.50/10 hpf for invasive bladder cancer. However, AgNOR and bcl-2 did not show significant specific or sensitive cut-off values to detect invasive bladder cancer cases.

DISCUSSION

We assessed the relationship of mitotic indices, AgNOR count, Ki-67 LI and anti apoptotic bcl-2 immunopositivity with the well established prognostic parameters, that is, tumor grade and stage. We found a significant strong positive correlation of mitotic indices and Ki-67 LI with bladder cancer grade and stage, which is explained by the significant increase in proliferative activity of tumor cells with muscle invasion. Ki-67 is a more accurate pro-

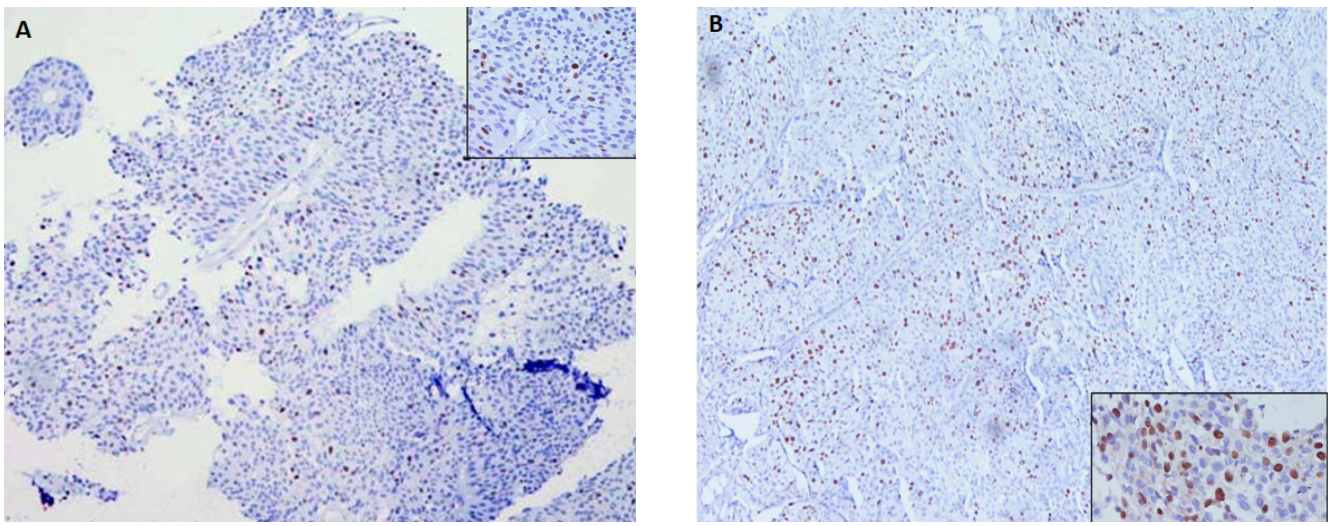

Figure 4. a) Low Ki-67 Labelling index in low grade papillary bladder cancer (Ki-67 immunostain, ×100) inset- higher magnification (×400). b) High Ki-67 Labelling index in high grade papillary bladder cancer (Ki-67 immunostain, ×100) inset- higher magnification (×400).

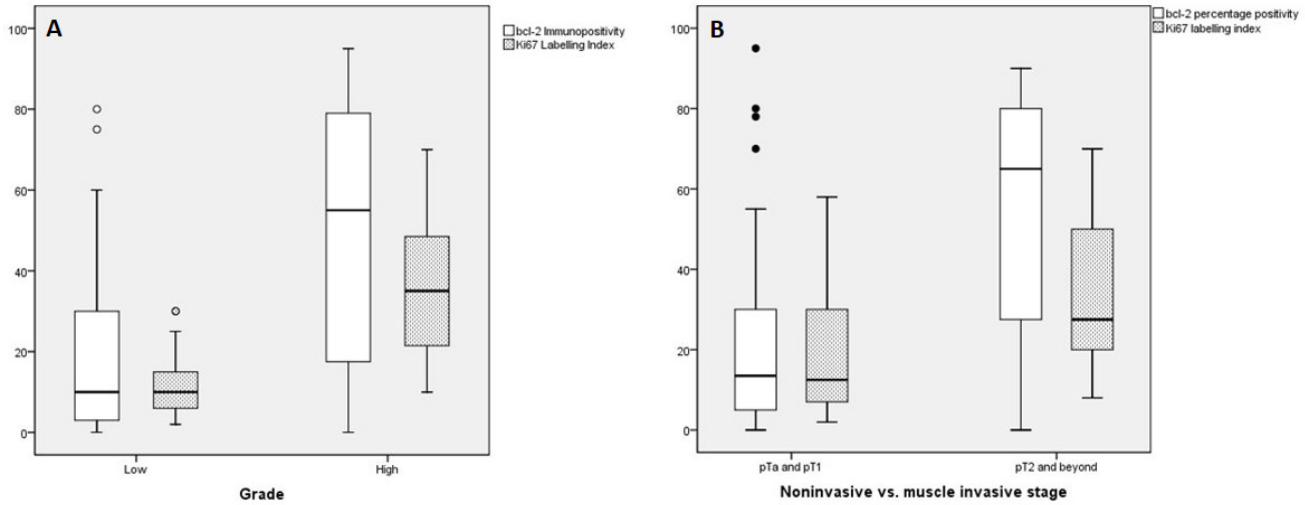

Figure 5. a) Box and whisker plot of bcl-2 and Ki-67 percentage immunopositivity with grade of pUBC showing higher values associated with high grade UBC. b) Box and whisker plot of bcl-2 and Ki-67 percentage immunopositivity in invasive (stage pT2 and beyond) and superficial (stage pTa and pT1) pUBC cases. The lines within boxes represent median value. The top and bottom of boxes (hinges) represent 25th and 75th percentiles of the data values. The T bars that extend from the boxes (whiskers) are expected to include 95% of the data (assuming normal distribution). The points represent outliers.

liferating index because it stains all the phases of cell cycle except G0. However, it requires standardized immunohistochemistry setup for valid results. Mitosis has long been employed as a simple way to measure proliferation on routine sections, but is limited to a phase of cell cycle with substantial inter-observer variability in its identification. Since area of a single high power field may vary up to 3-5 folds in different microscopes, M/V index is a more reliable and reproducible marker to assess proliferation. Lipponen and colleagues have also documented M/V index as a better independent prognostic indicator.[9] We found Ki-67 LI ≥ 32.5%, mitoses ≥ 14/10 hpf, ≥ 11.20 mitoses/mm^2 and ≥ 0.75 mitoses/100 tumor cells confirmatory for high grade bladder cancer (specificity = 100%). Gontero and colleagues have reported Ki-67 LI > 20% as predictor of recurrence in superficial low grade bladder cancer cases.[19] We found Ki-67 ≥ 59% and mitoses ≥ 36.50 /10 hpf 100% specific for invasive bladder cancer, which can have practical implications in tumor staging and management in situations where morphological evidence of muscle invasion is equivocal. Very limited studies done worldwide on Ki-67 in pUBC have reported the cut-off values, which have not yet been validated, nevertheless are practically useful in diagnosis, reflective of the relative trends (with their specificities and sensitivities) and serve as a guide in newer research.

Review of existing literature on AgNORs in bladder cancer yields confounding results regarding their correlation with grade, stage and prognosis.[13-5,20-22] We found a statistically significant positive correlation of mean AgNOR count with tumor grade, mitotic indices and Ki-67 LI. ROC analysis revealed mean AgNOR count ≥ 11.55 as 100% specific for diagnosis of high grade pUBC cases. However, no correlation was found with tumor stage or bcl-2 confirming the findings of previous researchers.[15,21,22] Small number of cases, aggregation of AgNORs leading to erroneous counts and technical factors related to staining and fixation may limit the practical utility of AgNOR in evaluation of an individual case. Measurement of AgNOR area per nucleus by quantitative image analyzer is a more accurate and objective method to reflect tumor behavior as it provides indirect measurement (ratio) of AgNOR proteins amount close to 3D evaluation.[23]

bcl-2 cytoplasmic positivity in superficial urothelium was a hallmark of pUBC cases while basal layer expression of bcl-2 was seen in normal urothelium and cystitis. Previous studies have reported bcl-2 positivity ranging from 2 % to 69% in bladder tumors. We found bcl-2 expression in 64.8% of pUBC cases. We found significantly higher non basal bcl-2 expression in high grade and muscle invasive tumors as compared to low grade and superficial pUBC, highlighting the role of bcl-2 up-regulation in pathogenesis and progression of bladder cancer.[7,24-28] Shift in bcl-2 expression from basal cells of non-neo-

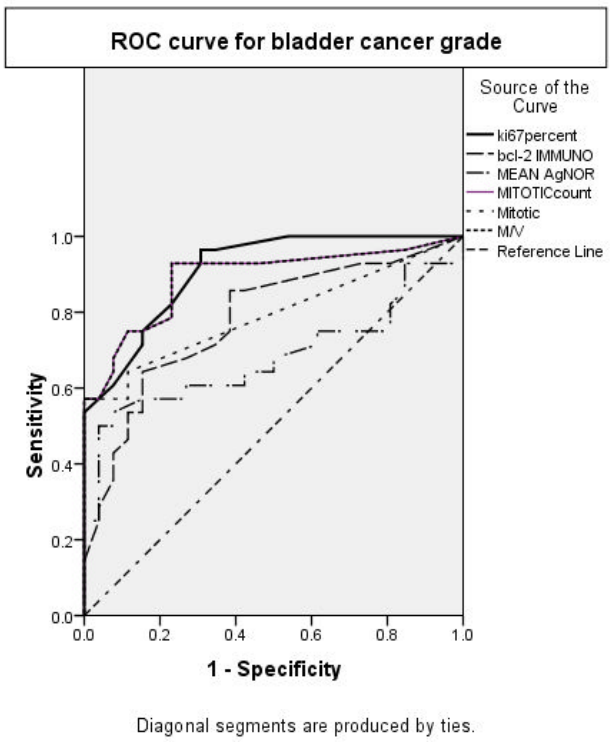

Figure 6. ROC curve of Mitotic indices, AgNOR count, bcl-2 and Ki-67 labelling index for bladder cancer grade.

plastic urothelium to superficial layers of bladder cancer, reflect deregulation of the control mechanisms, conferring a prolonged survival advantage and when superimposed by uncontrolled cell proliferation, leads to definite tumor progression. Higher incidence of Ki-67 and mitotic indices in bcl-2 positive tumors suggest that aggressive high grade bladder cancer cells with higher proliferative activity also have longer survival.

In contrast, few studies in literature have reported inverse[16,30,32] and no significant correlation[29,31,33] of bcl-2 with grade, stage and survival of bladder cancer. Controversial relationship of bcl-2 with clinicopathological parameters and survival in bladder cancer noted in these studies raises the need for further studies regarding the role of genes involved in the regulation of apoptosis in bladder cancer.

There were few limitations of our study. First, the sample size was small for uniform grade and stage wise distribution of cases. Second, the mitotic and AgNOR count are subjective, and we did not evaluate inter-observer variability in their interpretation. Third, since we retrospectively analyzed the archival blocks, follow up of the patients was not possible in our study. There is a need to apply these cut-off values in equivocal cases and follow them up in larger prospective studies to validate our results.

In conclusion, the three proliferative markers studied here correlated strongly with tumor grade. Mitotic indices and Ki-67 correlated with tumor stage, while AgNOR was not useful in evaluating muscle invasive bladder tumor cases. bcl-2 was found to correlate positively with both tumor grade and stage. All the proliferative markers correlated strongly with each other. Ki-67 and mitotic indices showed a positive correlation with bcl-2, but AgNOR did not show any correlation with bcl-2. Ki-67 $\geq$ 59% and mitotic count $\geq$ 36.50 per 10 hpf, if proven seem to be promising and reliable indicators to assess muscle invasion in superficial bladder biopsies and TURBT samples where muscle is not resected. Keeping in view, the cost, time and workload constraints, this may be of practical utility in developing countries, as of ours, where it is not feasible to do multiple repeat biopsies and patients are often lost to follow up. We suggest that such cases should be considered muscle-invasive, for all practical purposes. Also, absolute values of Ki-67 LI $\geq$ 32.5% , mitoses $\geq$ 14/10 hpf, $\geq$ 11.20 mitoses/mm^2, $\geq$ 0.75 mitoses/100 tumor cells and AgNOR $\geq$ 11.55 can increase the diagnostic confidence (specificity = 100%) for high grade bladder carcinoma situations where clear distinction between low and high grade is not possible on morphology alone. Thus, addition of these markers to the existing protocols may increase the objectivity and reliability for accurate diagnosis, patient management and tumor progression than the conventional grade and stage alone.

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CONFLICT OF INTEREST

None declared.

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