

## Role of morphometry and proliferative parameters in grading of urothelial neoplasms

Monika Sangwan<sup>1</sup>, Sunita Singh<sup>1</sup>, Santosh Kumar<sup>1</sup>, Sonia Chhabra<sup>1</sup>, Rajeev Sen<sup>1</sup>, Praveen Rana<sup>2</sup>, Shivani Malik<sup>1</sup>, Sonia Singh<sup>1</sup>, Ramesh Lamba<sup>3</sup>

<sup>1</sup>Pt.B.D. Sharma Institute of Medical Sciences, Rohtak, Haryana, India

<sup>2</sup>Govt. Medical College for Women, Khanpur Kalan, Sonapat, Haryana, India

<sup>3</sup>Community Health Centre, Kiloj, Rohtak, Haryana, India

**Citation:** Sangwan M, Singh S, Kumar S, Chhabra S, Sen R, Rana P, et al. Role of morphometry and proliferative parameters in grading of urothelial neoplasms. Cent European J Urol. 2015; 68: 37-44.

### Article history

Submitted: Aug. 4, 2014

Accepted: Dec. 13, 2014

Published on-line:

March 13, 2015

### Corresponding author

Monika Sangwan

Department of Pathology

Medical College

124001 Rohtak, India

phone: +91 946 633 6734

dr.monika1311@yahoo.in

**Introduction** Mean nuclear area of 10 nuclei (MNA-10), mitotic activity index (MAI) and Ki-67 are highly reproducible and can be routinely used as adjuncts to histopathological grading in classifying tumors. Assays of these biomarkers are non-invasive, rapid, easy to perform, more objective and accurate, with high sensitivity and specificity, and correlate well with tumor grade.

**Material and methods** This study was conducted at the Department of Pathology PGIMS, Rohtak on 50 cases, of which 25 cases were high-grade, 15 low-grade, 6 Papillary Urothelial Neoplasm of Low Malignant Potential and 4 reactive lesions as per the 2004 ISUP/WHO classification. MNA-10, MAI and Ki-67 immunohistochemical analysis were performed on stained sections.

**Results** The age of the patients varied from 35 to 87 years. Male: female ratio was 3.5:1. The mean MNA-10 ( $\mu\text{m}^2$ ) for High Grade Malignant Potential was  $104.52 \pm 25.64 \mu\text{m}^2$ , which was significantly higher than in PUNLMP ( $47.64 \pm 10.23$ ) and LMP ( $51.57 \pm 15.66$ ). MAI (/10 HPF) showed an increasing trend from reactive lesions to HMP, with a mean of ( $3 \pm 1.16$ )/10 HPF to ( $21.36 \pm 5.31$ )/10 HPF respectively. Ki-67 labelling index, a proliferative marker, revealed increasing trend lowest with reactive lesions ( $10 \pm 2.83\%$ ) and highest in high grade tumors ( $65.96 \pm 14.44$ ). Spearman's correlation showed maximum correlation between MAI and Ki-67 and the increasing grade of tumor.

**Conclusions** MNA-10 in combination with Ki-67 and MAI was found to be stronger than MNA-10 alone. MAI has high reproducibility in differentiating low and high grade, with simple assessment in paraffin embedded sections allowing adequate histopathological analysis and visualization of proliferating cells simultaneously. This multivariate grading model should be applied in routine grading to overcome interobserver variability and to increase reproducibility of grading.

**Key Words:** morphometry <> mitotic activity index <> urothelial tumors <> proliferative parameters

## INTRODUCTION

Urinary bladder cancer is the 13<sup>th</sup> leading cause of cancer-related death among men and women worldwide. An estimated 386,300 new cases of bladder cancer occurred in 2008, making it the 9<sup>th</sup> most common cause of cancer worldwide. The highest incidence rates are found in Western countries [1], but incidence is increasing in India. According to recent

statistics from the Indian Cancer Registry, bladder cancer is 9<sup>th</sup> most common cancer recorded in India, contributing 3.9% of all cancer cases, irrespective of gender [2]. Much controversy, however, surrounds the grading of bladder tumors [3]. Arising from the need to develop a commonly accepted classification system that pathologists, urologists, and oncologists could use, various new classification systems have been proposed, including the World Health Organi-

zation (WHO) 1973 and the International Society of Urological Pathology (ISUP) 1998 and 2004 classifications. According to 1998 WHO/ISUP, 1999 WHO Blue Books and the 2004 WHO classifications, the noninvasive urothelial neoplasms have been divided into three major subgroups – flat, endophytic and papillary. Papillary urothelial neoplasms have been further categorized as urothelial papilloma (UP), inverted papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma, and high-grade papillary urothelial carcinoma [4].

Despite the introduction of the new 2004 WHO/ISUP classification, the grading of urothelial carcinoma remains difficult and subjective [5]. The literature indicates that there is still much debate as to whether the 2004 classification should be used exclusively and whether the 1973 WHO form should be abandoned. Reporting both grades has been recommended [3].

The best grading system should not only be easy to apply, but also allow for division of tumors into groups with different biologic characteristics that correlate with different clinical outcomes. Subjective grading criteria used in both grading systems are not perfectly reproducible. Interobserver reproducibility of grading may be as low as 60–75%. Therefore, the need for objective grading is high, which would increase the reproducibility of a classification system [6].

Proliferation markers, including molecular markers, mitotic activity index and the quantification of nuclear features using morphometry may have a valuable role in further refinements of the classification system, and could be correlated very well with histologic grade in bladder cancer, in addition to being highly reproducible [7]. Such a multiparameter study is very useful as these features are reliable, relatively cheap and easy to assess, and discrimination between different grades may increase when these quantitative and proliferative parameters are used as an adjunct to routine histopathological grading, all with an increase in reproducibility.

The mean profile area of the 10 largest nuclei (MNA–10) found in a histological tumour section has proved to be valuable as an independent marker for grade and prognosis [9]. Additional studies demonstrate a significant correlation between the Ki–67 index and the tumor grade and stage of urinary bladder cancer [8]. It has been noted that Ki–67 offers good discriminatory power between Low Malignant Potential cases and carcinoma [9]. Mitotic activity index (MAI) could be applied in the routine examination of urothelial tumor diseases with increased risk of malignancy, primarily because it is quick and easy to apply, and secondly because of its high degree of fidelity and objectivity [9].

Thus quantitative nuclear parameters (MNA–10 nuclei) and proliferative parameters (Ki–67 and MAI) are accurate, well reproducible, easy to assess, progression predictors and can aid in grading all patients with urothelial cell carcinomas. The purpose of our study on transitional cell carcinomas (TCC) of the urinary bladder is to develop a reproducible method for grading that is simple and robust, and may thus be used in daily patient care.

## MATERIAL AND METHODS

The present study was conducted in the Department of Pathology Pt. B.D. Sharma, PGIMS, Rohtak. The study group was confined to fifty cases of urothelial neoplasm of different grades obtained either by transurethral resection or by biopsy performed at the Departments of Urology. The tissue was fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin wax. Haematoxylin and eosin (H&E) stained 4  $\mu$ m histological sections were used. All fifty cases of TCCs were evaluated according to the World Health Organisation/ISUP 2004 classification and were classified as reactive hyperplasia, papilloma, PUNLMP, LMP (Low Malignant Potential) and HMP (High Malignant Potential). The worst differentiated areas were taken into consideration for mitotic counts and MNA–10 measurements, avoiding the necrotic, damaged, inflamed, and benign or lower grade parts of the section.

### Quantitative analysis

MNA–10 measurement was performed on H&E stained sections for the subjectively 10 largest nuclei using an Olympus BX 51 microscope at a total magnification of 400x, and at a magnification of 400x on the host computer, processing was done by image-analyzing software (Microsoft Image Pro-Plus version 6.3).

### Proliferative parameters

MAI was assessed by counting mitotic figures using well-established criteria. Where histological interpretation was in doubt, a mitosis was not registered.

### Immunoquantitation

Ki–67 immunoquantitation was performed using light microscopy at 400x magnification. The percentage of tumor cells with nuclear staining was determined by counting at least 1000 tumor cells in the ten selected fields displaying the highest immunoreactivity.

## Procedure for Immunohistochemical (IHC) staining [10]

IHC for Ki-67 was performed by mounting 4–5  $\mu\text{m}$  sections on slides coated with suitable tissue adhesive, deparaffinization of sections in xylene and rehydration through graded alcohols; antigen retrieval was done in a pressure cooker. The sections were rinsed in Tris-buffered saline (TBS). Endogenous peroxidase was blocked using a peroxidase block. After washing in TBS, the slides were incubated with the protein block. The wash was repeated and diluted primary antibody was applied for 60 minutes, washed, and incubated with post-primary block for 30 minutes. The slides were rinsed again with the buffer before being incubated with polymer for 30 minutes, further washed and incubated in Diaminobenzidine (DAB) solution for 10 minutes. The slides were rinsed a final time in TBS and transferred to running water. Counterstaining was done with Mayer's hematoxylin. Finally, the sections were dehydrated in graded alcohols and xylene, and mounted with distrene 80 dibutyl phthalate xylene (DPX).

The results are interpreted by the brown color of proliferating nuclei. Positive and negative controls were used.

## Interpretation and statistical analysis

The stained sections were examined and each lesion was graded according to the 2004 WHO/ISUP classification. These grades were correlated and compared with quantitative morphometric (MNA-10 nuclei) and proliferative (Mitotic Activity Index and Ki-67 labelling index) parameters with routine histopathological grade. New grading was done by using these three parameters as an adjunct to the routine histopathological grade.

## Data analysis

The values of MNA-10 nuclei ( $\mu\text{m}^2$ ), Mitotic Activity Index (/10 HPF) and Ki-67 labelling index (%) were entered into a master spreadsheet (Microsoft Excel) and analyzed using SPSSv20 software. Descriptive statistics (mean, standard deviation, range, frequency distribution, percentages) were applied wherever appropriate. Spearman's correlation test was used to determine the correlation between various variables. Graphical representation was done wherever necessary. All the values showed significant correlation with each other and with an increase in the grade of the urothelial neoplasm. The P-value was significant ( $<0.05$ ) for the grading according to these parameters.

## Observations

Table 1 shows MNA-10 in different grades of tumor and a comparison with the initial and final grade, using quantitative and proliferative parameters.

Table 2 gives the ranges and means with standard deviation of MAI/10 HPF, in addition to the comparison with initial and final grade after using quantitative and proliferative parameters.

Table 3, presents the Ki-67 labelling index in the various grades of tumor. An increasing trend with an increase in the grade of the tumor is observed.

In Table 4, a comparison is made between the routine histological grading done using the 2004 WHO/ISUP classification and the grading performed in our study according to the quantitative and proliferative parameters. Discrepancy arose in seven cases giving an overall correct classification of 86% with the 2004 WHO/ISUP classification.

**Table 1.** Comparison of MNA-10 ( $\mu\text{m}^2$ ) in various grades of tumor

Mean nuclear area 10 nuclei (MNA-10 $\mu\text{m}^2$ )		
Category	Range	Mean $\pm$ SD
Reactive	32.2–40.2	37.10 $\pm$ 3.80
PUNLMP	29.8–54.6	47.64 $\pm$ 10.23
LMP	39.4–58.2	51.57 $\pm$ 15.66
HMP	69.8–159.2	104.52 $\pm$ 25.64

**Table 2.** Comparison of MAI (/10 HPF) in various grades of tumor

Mitotic activity index (MAI/10 HPF)		
Category	Range	Mean $\pm$ SD
Reactive	2–4	3 $\pm$ 1.16
PUNLMP	2–4	3.29 $\pm$ 1.49
LMP	5–14	6.42 $\pm$ 1.68
HMP	15–30	21.36 $\pm$ 5.31

**Table 3.** Comparison of Ki-67 in various grades of tumor

Ki-67 labelling index (%)		
Category	Range	Mean $\pm$ SD
Reactive	06–12	10.00 $\pm$ 2.83
PUNLMP	02–12	10.00 $\pm$ 5.03
LMP	15–38	26.67 $\pm$ 7.36
HMP	46–90	65.96 $\pm$ 14.44

**Table 4.** Comparison of grading on the basis of histopathology alone and according to MNA-10, MAI and Ki 67

Grading on the basis of histopathology	Revised grade according to (MNA-10, MAI and Ki-67)				Total
	Reactive	PUNLMP	LMP	HMP	
Reactive	04	00	00	00	04
PUNLMP	00	05	01	00	06
LMP	00	02	10	03	15
HMP	00	00	01	24	25
Total	04	07	12	27	50

**Table 5.** Distribution of cases of LMP with maximum variation (5 cases out of 15)

Case	Histopathological diagnosis	MNA-10 ( $\mu\text{m}^2$ )	MAI (/10 HPF)	Ki-67 (%)	Final grading
1.	LMP	75.3	15	38	HMP
2.	LMP	95.0	17	48	HMP
3.	LMP	43.0	04	06	PUNLMP
4.	LMP	41.3	03	10	PUNLMP
5.	LMP	50.5	25	64	HMP

Finally, Table 5 shows the distribution and variation in five out of 15 cases graded as LMP in routine reporting, and their subsequent change into PUNLMP (2), LMP (10) and HMP (03) after using the quantitative and proliferative parameters

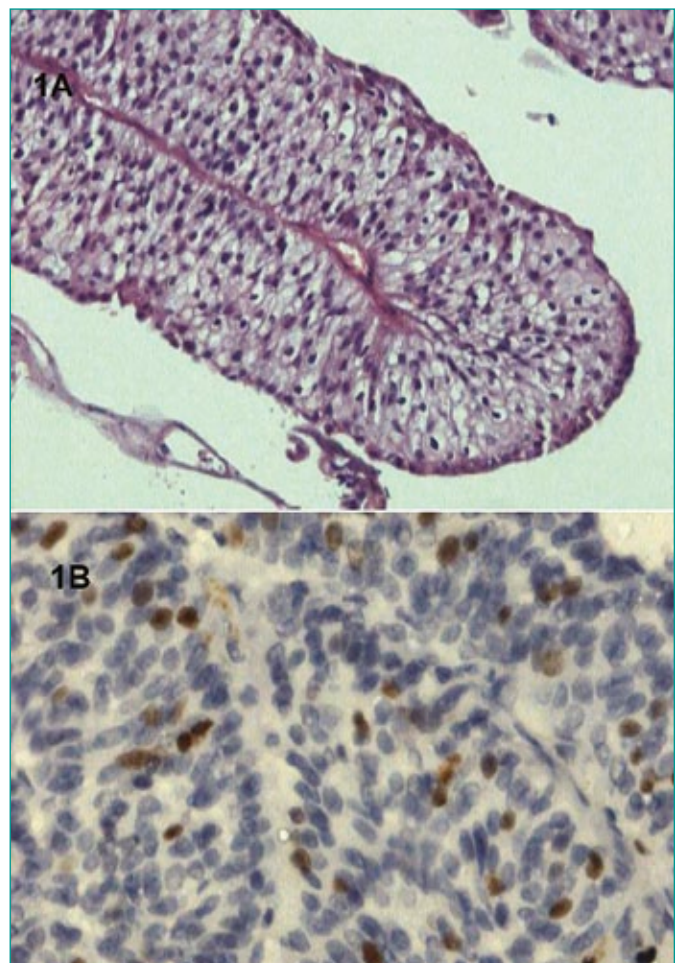
## DISCUSSION

Bladder cancer is morphologically heterogeneous. Over ninety percent of bladder cancer cases are urothelial (transitional cell) carcinomas, whereas primary squamous cell carcinoma, adenocarcinoma and other tumors are less common [11]. The pathologic grade is usually critical in determining the clinical management and prognosis of patients. Several classifications of noninvasive neoplasms have been proposed. However, current grading systems have long been a source of controversy. The previously widely-used 1973 WHO three-tiered system (grade 1 to 3) was converted into the two-tiered (low grade [LG] vs. high grade [HG]) 2004 WHO classification or 1998 WHO/International Society of Urological Pathology classification, which delineate a set of specific and standardized architectural and cytologic criteria for each grade [12]. The 2004 system introduced a new category, papillary urothelial neoplasm of low

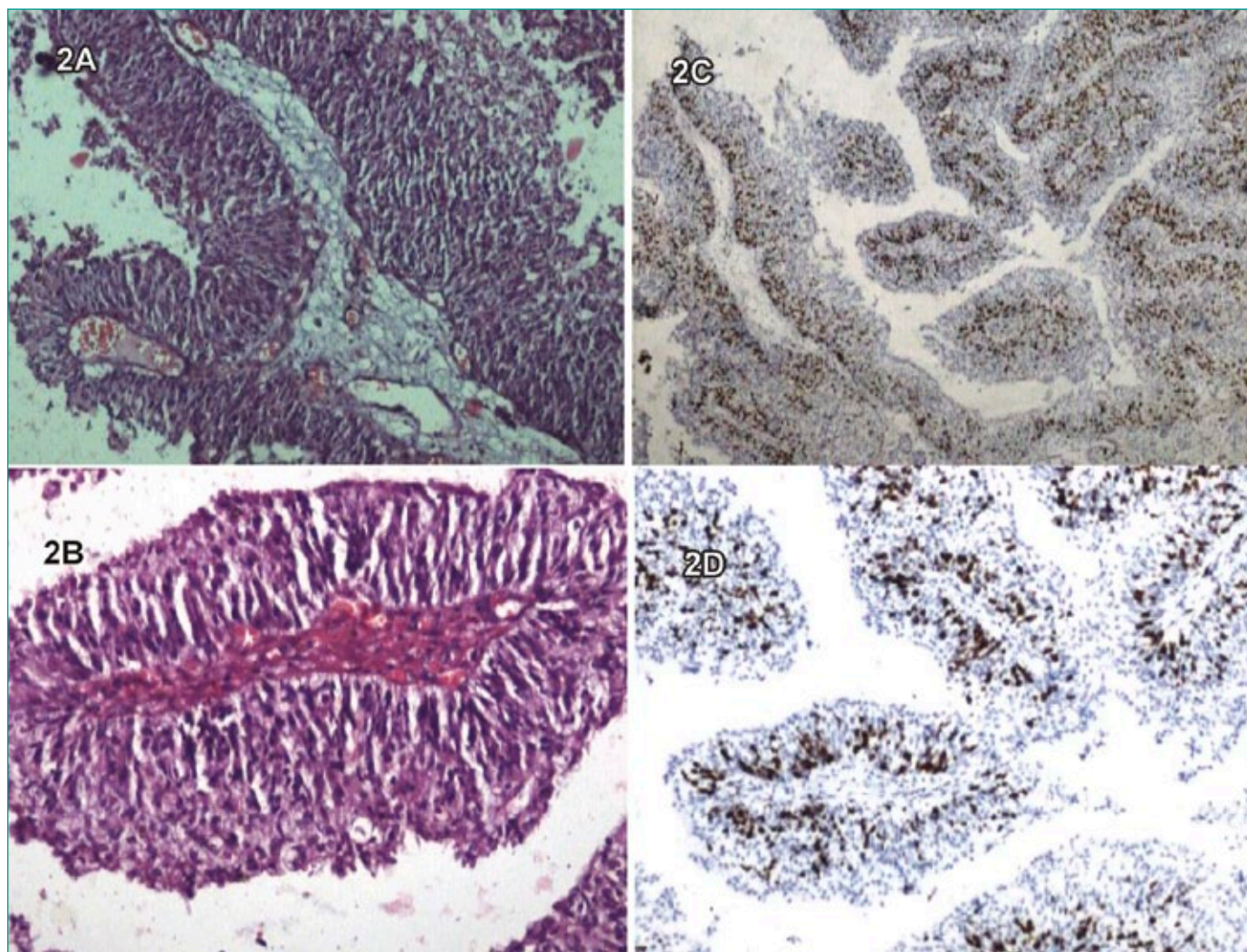
malignant potential (PUNLMP), to encompass cases with even lower potential for progression than typical LG carcinomas [4].

Multiple studies assessing the 2004 WHO classification have been performed, but universal acceptance of this grading system has not been achieved because of persistent questions regarding its validity and usefulness [13]. High interobserver variability has been seen, especially in distinguishing PUNLMPs from LG carcinomas [14, 15] (Figure 1 & 2). Considering the heterogeneity and broad range of morphologic features in both LG and HG papillary carcinomas, one key issue still awaiting investigation is whether the two-tiered morphologic grading scheme reliably and sufficiently reflects the biology and clinical behavior of these tumors and, more practically, whether there are adjunct immunohistochemical or molecular markers that could be helpful in the classification (Figure 2, 3).

Our study of 50 cases of bladder lesions included 4 reactive lesions. The mean patient age was 60 years. It is rare in people younger than 50; the median age



**Figure 1.** PUNLMP (1A) H&E; 200X (1B) Ki-67; 100x.



**Figure 2.** Low grade tumor (2A) H&E; 200x, (2B) H&E; 400x, (2C) Ki-67; 200x, (2D) Ki-67; 400x.

at time of diagnosis is 70 years for each gender [2]. The age (60–69 years) of diagnosis was found to be similar in various studies from Asian, Middle East and Western countries [15–20]. The incidence of TCC increases with age and is higher among males than females, with a ratio of 3.5:1. This is similar to the ratio (4:1) reported in The United States [18] and globally [1, 18]. However, higher male:female ratios, varying from 6:1 to 8.6:1, have also been reported in literature [2, 15, 17, 21].

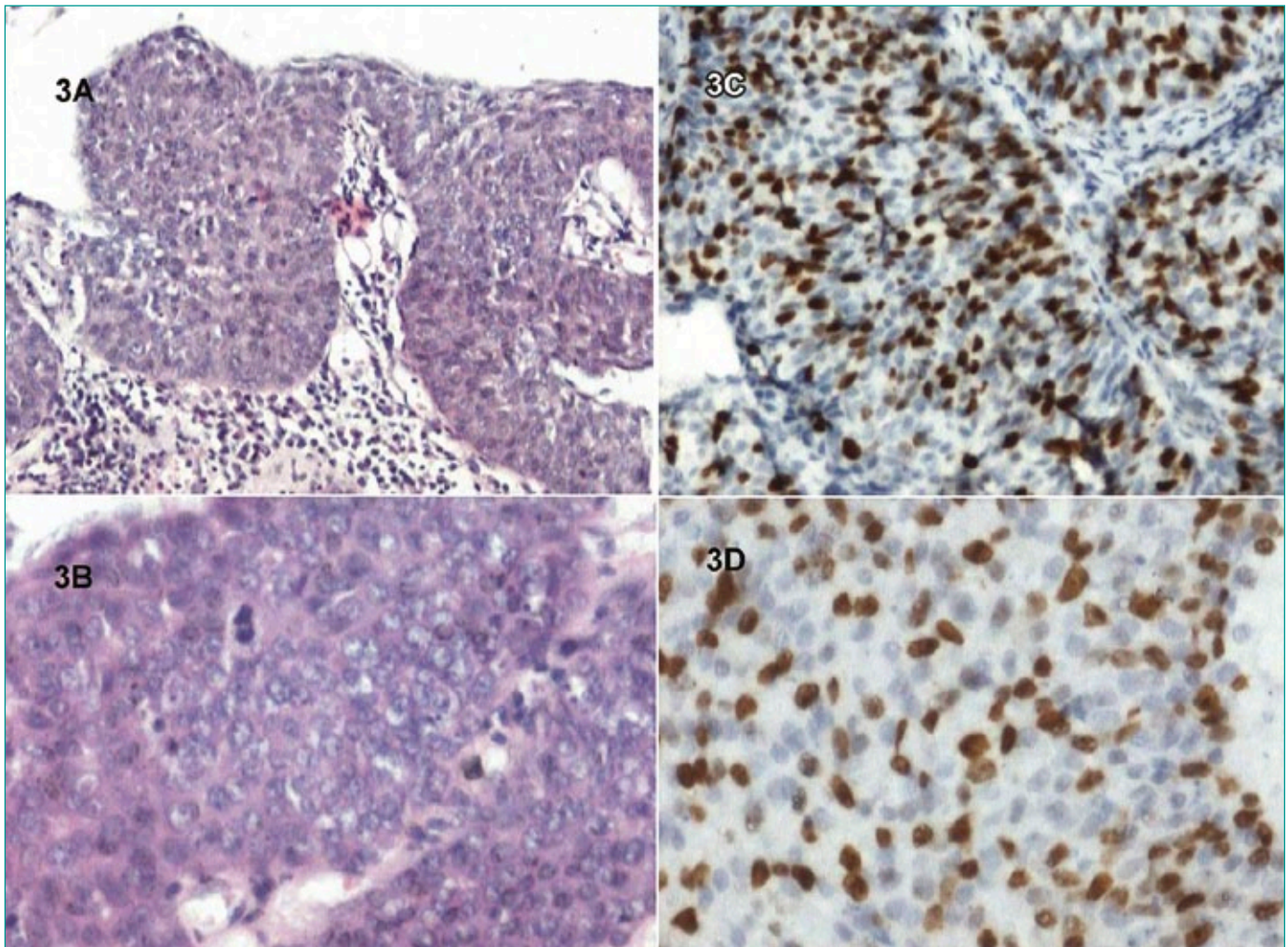
According to the 2004 WHO classification, 50 bladder biopsies were classified histopathologically as reactive hyperplasia, papilloma, PUNLMP, LMP and HMP. The majority were high grade (50%), 30% were low grade and 12% were PUNLMP.

The tumors varied in size from 1 to 2.5 cm in LMP and PUNLMP, and to more than 2.5 cm in HMP. We identified lesions of, the lateral wall (42%), posterolateral wall (28%), posterior wall (16%) neck

(10%) and dome (4%). All tumors of the dome and neck were HMP. The posterior wall showed a relatively high incidence for HMP (50%) and the lateral or posterior wall were involved relatively in all grades [22, 23, 24].

However due to subjectivity, the reproducibility of the grading system is low [25, 26]. According to Epstein et al., morphometric grading into two groups support WHO/ISUP grading of TCC as low grade and high grade urothelial neoplasm [27]. In our study, the values of the mean nuclear area of the 10 largest nuclei of HMP are significantly higher than in PUNLMP and LMP, but there is considerable overlap in the range between reactive and PUNLMP and LMP (Table 1). So MNA-10 alone could be significantly used in distinguishing between low and high grades.

Cell proliferation is related to histological grading as well as prognosis. MAI (/10 HPF) in HMP ranged



**Figure 3.** High grade tumor (3A) H&E; 200x (3B) H&E; 400x (3C) Ki-67; 200x (3D) Ki-67; 400x.

from 15 to 30/10 HPF (mean  $21.36 \pm 5.3/10$  HPF) and in LMP, from 5 to 14/10 HPF (mean  $6.42 \pm 1.68/10$  HPF) (Table 2). We found MAI to be significantly lower, 2 to 4/10 HPF, in both PUNLMP (mean  $3.29 \pm 1.49/10$  HPF) and reactive lesions (mean  $3 \pm 1.16$ ). Various authors have also documented similar findings, and it has been suggested that grading of superficial TCC could be based on M/V Index (Volume corrected mitotic index) alone; it is the best histological variable for grading and prognosis [28, 29, 30]. Ki-67 is a nuclear protein and is used to predict proliferative activity and thus biological aggressiveness of the tumor [31]. In our study Ki-67 labelling index showed an increasing trend with an increase in grade (Figure 1, 2, 3). Although an overlap was observed in PUNLMP and reactive lesions, PUNLMP, LMP and HMP showed significant correlation between histological grade and Ki-67 positivity (Table 3). Our findings are in concordance with studies conducted by Cina et al. [32]. Fontana et al. [33]. High tumor

proliferation or Ki-67 labelling index may increase the value of the WHO grading system as well as predictor of stage progression [8, 34, 35].

When all three parameters were combined with the routine histological grade, there were discrepancies in the grading of seven cases. Only 86% cases were graded correctly using only the 2004 ISUP/WHO classification (Table 4). Maximum variation was seen in low grade tumors.

The grades of five from a total of 15 LMP cases were revised after using quantitative and proliferative parameters (Table 5). Out of these five cases, two were revised to high grade on the basis of all three parameters that were found to be within the range of high grade. Murphy et al. concluded that discrepancies were higher in discriminating PUNLMP and LMP and that quantitative and proliferative parameters had high reproducibility and could be used as an adjunct to routine histopathology in differentiating various grades [13].

One case of LMP was also upgraded to HMP on the basis of high proliferation, with an increase in MAI and Ki-67, but where MNA-10 nuclei remained within the range. Zainab et al. concluded that morphometric analysis should be used cautiously in dealing with TCC, especially when trying to discriminate between grade II and III [36]. Two cases of LMP were downgraded to PUNLMP, as MAI and Ki-67 were within the range for PUNLMP. Shim et al. proposed a scoring system suggesting that mitosis may be the best diagnostic parameter for reproducibility of PUNLMP [37].

One case of PUNLMP was revised to low grade. MAI and Ki-67 again proved to be more reproducible in differentiating between PUNLMP and LMP.

One case of HMP out of 25 was downgraded to LMP. MNA-10 was within the range of HMP, but mitotic activity and Ki-67 were very low. Again in this case MAI and Ki-67 alone were much more reproducible than using only MNA-10 nuclei.

MNA-10 can be used to differentiate low grade from high grade lesions. However, its reproducibility in grading is lower than MAI and Ki-67, which are highly reproducible as an adjunct to histopathological grading. Bol et al. concluded that the discriminating

power of MNA-10, when combined with Ki-67 and MAI, was found to be stronger than that of MNA-10 alone [6]. Pich et al. also observed many overlaps of values between G1 and G2 tumors, but no overlapping was found between G3 and G1/G2 tumors [34].

## CONCLUSIONS

MNA-10 nuclei alone could be used significantly in distinguishing low and high grades and its use is reproducible. An advantage of MAI is high reproducibility and simplicity of assessment as it can be determined from paraffin embedded sections and also allows for visualization of proliferating cells and adequate histopathological assessment. MNA-10 in combination with Ki-67 and MAI was found to be stronger than MNA-10 alone. MAI and Ki-67 are reproducible and can be routinely used as an adjunct to histopathological grading in classifying tumors. This multivariate grading model should be applied in routine grading to overcome interobserver variability and increase the reproducibility of grading. These biomarkers are non-invasive, rapidly and easily assessed, more objective and accurate with high sensitivity and specificity, correlating well with the grade.

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