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ORIGINAL ARTICLE

Comparative Application of Different Substaging Techniques for Non-Muscle Invasive Urothelial Carcinoma

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Abstract

To evaluate the diagnostic performance and clinical significance of 4 systems of substaging cases with non-muscle invasive urothelial bladder carcinoma. In addition 4 cutoff measures were evaluated for prediction of muscularis-mucosa invasion. Four substaging systems were applied to 57 NMIBC cases to assess which of these reported methods correlates best with recurrence and progression. On univariate regression analysis patients having tumor size more than 3 cm, solid tumor architecture, high grade, substage B, substage T1e, substage ROL 2 and Tumor depth more than 1 mm were associated with higher recurrence. On multivariate analysis all the four substaging systems, tumor size, grade and tumor type had significant prognostic value for recurrence. Regarding progression only the metric substaging method was associated with tumor progression ($p = 0.04$). However, on univariate and multivariate regression analysis none of the substaging systems showed prognostic significance and only solid tumor architecture and CIS had significant prognostic value for tumor progression. The ROC curve analysis showed that 1 mm depth of invasion had the best accuracy for detection of muscularis-mucosa invasion (80.2%). Using 1 mm cutoff in measuring the depth and 0.5 mm for the diameter of infiltration may provide clinically relevant information to guide a more personalized therapy for NMIBC. Inclusion of both measures in addition to other histopathologic variables may aid in the development of a scoring system.

Keywords Substaging · Non-muscle invasive · Urothelial carcinoma · Urinary bladder

Introduction

Bladder carcinoma (BC) is the most common malignancy involving the urinary system and the ninth most common malignancy worldwide [1]. Nearly 70% of newly diagnosed urinary bladder carcinoma are non-muscle invasive (NMIBC) which are usually treated by transurethral resection bladder tumor (TURBT) with or without intravesical immunotherapy or chemotherapy. Despite complete TURBT, high rate of recurrence is encountered; as early recurrence within 1 year occurs in 40% of cases and 70% of patients suffer from recurrence within 5 years [2].

NMIBC is a heterogeneous group with varying outcomes and up to 50% of cases progress to muscle invasive bladder cancer (MIBC) and one third metastasize. Early radical cystectomy may be recommended in high risk patients. However, it would be over treatment for non progressor cancers [3].

Further identification of prognostic morphologic information for NMIBC patients is much needed. One of the pathological details most often suggested is substaging of T1 cases. Many different methods have consequently been proposed in the past few years, but none has been yet considered satisfactory [4, 5].

The most extensively studied sub-staging system is based on invasion of the muscularis mucosa, which is a discontinuous layer of smooth muscle bundles accompanied by large blood vessels plexus and situated, approximately, midway between the urothelium and the muscularis propria. Tumors

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found within the lamina propria above the muscularis mucosa are sub-staged as (T1a) while those within or beyond the muscularis mucosa are sub-staged as (T1b) and incline worse prognosis [6]. With another prospective, some studies investigated depth of invasion measured by micrometer with variable cutoffs (1.5 mm and 3 mm) and found significant difference in progression rate [7, 8].

Some research groups established a different clinically significant method for classification named the metric substaging which is based on measuring the invasive diameter; tumor with only single spot <0.5 mm invasion is called T1 microinvasive (T1 m) and those having diameter ≥ 0.5 mm or multifocal tumors are called T1 extensive invasive (T1e) with worse prognosis [9, 10]. Another study suggested the use of 1 mm cutoff instead of 0.5 mm tumor diameter and called this substaging approach ROL (Rete Oncologica Lombarda) system [4] .

Table 1 Demographic and clinical history of the studied cohort

Variable	Category	*n* = 57
Age in years	• Mean ± SD	63.58 ± 11.4
	• Median (Range)	46 (30–82)
Sex	• Female	7 (12.3%)
	• Male	50 (87.7%)
Tumour Size	• < 3 cm	20 (35.12%)
	• ≥ 3 cm	37 (64.9%)
ROL substaging system	• ROL1 ≤ 1 mm	22 (38.6%)
	• ROL2 > 1 mm	35 (61.4%)
Metric substaging system	• ≤ 0.5 mm	16 (28.1%)
	• > 0.5 mm	41 (71.9%)
MM invasion Level	• A	28 (49.1%)
	• B	29 (50.9%)
Muscularis Mucosa	• Present	43 (75.4%)
	• Absent	14 (24.6%)
Tumour Depth/mm	• Mean ± SD	1.31 ± 0.1
	• Median (Range)	1 (0.01–3.79)
Tumour Depth Category	• ≤ 1 mm	27 (47.4%)
	• > 1 mm	30 (52.6%)
Multiplicity	• Solitary	34 (59.6%)
	• Multiple	23 (40.4%)
Tumour Architecture	• Solid	14 (24.6%)
	• Papillary	43 (75.4%)
Tumour Grade	• Low	23 (40.4%)
	• High	34 (59.6%)
CIS	• Present	8 (14%)
LVI	• Present	6 (10.5%)
Recurrence	• Present	31 (54.4%)
Progression	• Present	7 (12.3%)
Death	• Present	4 (7%)

In the current study we compared 4 substaging systems in addition to other histopathologic parameters to assess which of these reported methods correlates best with the prognosis of patients with NMIBC. In addition 4 cutoff measures were evaluated for prediction of muscularis-mucosa invasion.

Patients and Methods

This is a prospective study that included 57 patients who were diagnosed with NMIBC in the period from July 2016 to January 2018. All patients were graded according to 2004 World Health Organization (WHO) grading system and staged according to 2010 American joint Committee of cancer staging scheme. None of the patients had a history of urothelial carcinoma or were previously treated by chemotherapy or radiotherapy.

All patients had 2nd TUR (within 2–6 weeks) to confirm NMIBC status and ensure complete resection and were managed with 6 weekly doses of BCG and follow up was done by clinical monitoring, new imaging if needed and cystoscopy every 3 months. Mean follow up (FU) period was 15.75 ± 6.3 months, Median FU period was 14 (3–30) months.

Histopathological Assessments

H&E stained slides were examined and all selected cases had muscularis propria detected in the slides for proper staging. The following characteristics were examined:

1- Muscularis mucosae invasion: (pT1a = above the muscularis mucosae; pT1b = below or within the muscularis mucosae). In cases that muscularis mucosae could not be identified, localization of the invasive tumor in relation to vascular plexus was evaluated instead.
2- Maximum millimetric depth of invasion: This measurement represented the maximum tumor depth and invasion into the lamina propria. It was measured from the basement membrane of the covering epithelium to the deepest invasive tumor cells. Each TURBT fragment containing tumor was measured separately and the greatest depth of tumor invasion was used for the analysis. When the mucosa was not present or specimens were not oriented, the depth of invasion was measured from the shortest distance in invasive tumor foci [7].
3- Millimetric diameter of invasive focus: We applied 2 methods; first the metric substaging method: T1 m (Single focus of invasion >1HPF using objective 40×, ocular 10×/field 22, diameter 0.55 mm which correspond to 0.5 mm thickness of invasion). T1e (multifocal tumor or tumor invade more than 1 HPF of lamina propria) [9].

Also we applied ROL substaging method: ROL1(single focus of invasion >1 PF using objective

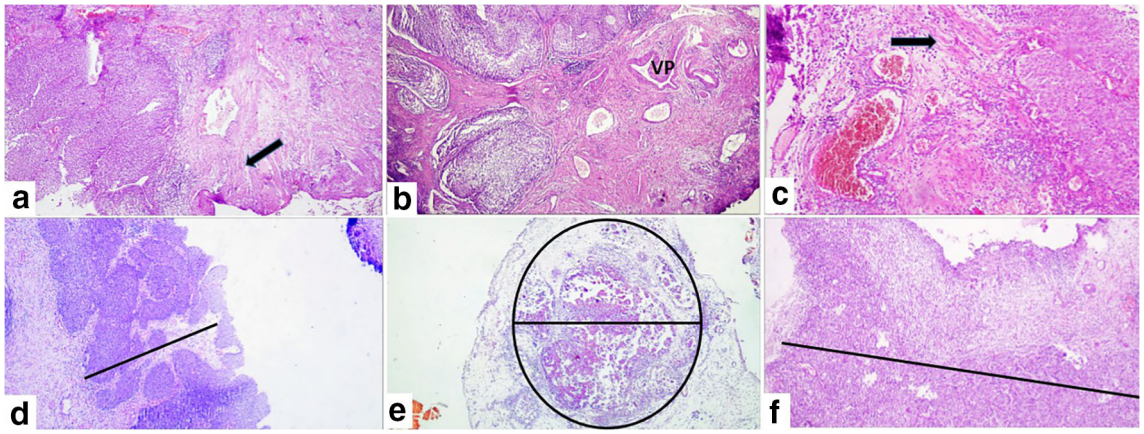

Fig. 1 **a** Hematoxylin and eosin stained image showing urothelial carcinoma infiltrating the lamina propria above the level of muscularis mucosa (arrow) (×40). **b** Urothelial carcinoma infiltrating the lamina propria at the level of vascular plexus (vp) ((×40). **c** Urothelial carcinoma infiltrating the muscularis mucosa (arrow) (×100). **d** Measuring depth of invasion from the basement membrane of the covering epithelium to the deepest invasive tumor cells (×40). **e** In badly oriented samples the depth of invasion was measured from the shortest distance of invasive tumor foci (×40). **f** Measuring the diameter of invasive urothelial carcinoma infiltrating the lamina propria (×40)

20×, ocular 10×/field 22, diameter 1.1 mm which correspond to 1 mm thickness of invasion), and ROL2 (multifocal tumor or tumor invade more than 1 mm thickness of lamina propria) [4].

4- Tumor stage and grade.
5- Coexisting CIS.
6- Presence or absence of lymphovascular invasion (LVI).
7- Tumor architecture (papillary versus solid).

These parameters were correlated with recurrence and progression. Recurrence was defined as the detection of any subsequent urothelial neoplastic lesion. Progression was defined as recurrent disease involving the muscularis propria (T2 or higher).

Table 2 Clinical history and demographics by disease recurrence status

Parameter	Category	Non-recurrent (no. = 26)	Recurrent (no. = 31)	*P* value
Age/years	• Mean ± SD	65.46 ± 9.1	62.00 ± 12.9	= 0.241*
Sex	• Female • Male	3 23	4 27	= 0.601**
Tumour Size	• < 3 cm • ≥ 3 cm	12 14	8 23	= **0.024****
ROL System	• ROL1 ≤ 1 mm • ROL2 > 1 mm	16 10	6 25	= **0.001****
Metric substaging	• ≤ 0.5 mm • > 0.5 mm	12 14	4 27	= **0.005****
MM invasion level	• A • B	22 4	6 25	< **0.001****
Tumour Depth	• Mean ± SD	0.67 ± 0.2	1.86 ± 0.2	< **0.001***
Tumour Depth Category	• ≤ 1 mm • > 1 mm	20 6	7 24	< **0.001****
Multiplicity	• Solitary • Multiple	17 9	17 14	= 0.296**
Tumour Architecture	• Solid • Papillary	1 25	13 18	= **0.001****
Tumour Grade	• Low • High	18 8	5 26	< **0.001****
CIS	• Present	3	5	= 0.458***
LVI	• Present	0	6	= **0.018*****

*T-test was used to compare the mean difference between groups

**Chi-square test was used to compare the proportion difference between groups

***Fisher's Exact test was used to compare the proportion difference between groups

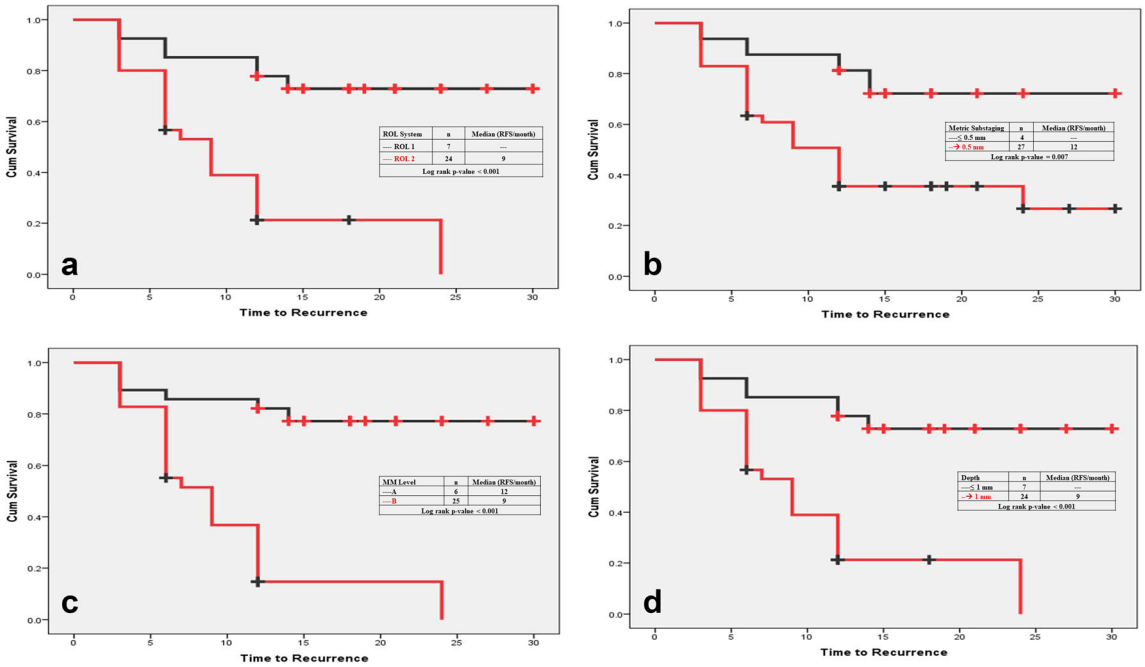

Fig. 2 **a** Kaplan-Meier survival plot of patient recurrence dependent on tumour diameter (cutoff, 1 mm) ($p < 0.001$, log rank test). **b** Kaplan-Meier survival plot of patient recurrence dependent on tumour diameter (cutoff, 0.05 mm) ($p = 0.007$, log rank test). **c** Kaplan-Meier survival plot of patient recurrence dependent on muscularis mucosa invasion ($p < 0.001$, log rank test). **d** Kaplan-Meier survival plot of patient recurrence dependent on tumour depth of invasion (cutoff, 1 mm) ($p < 0.001$, log rank test)

Ethical Considerations

The nature of the study was explained to all participants and a consent form was obtained before enrollment into the study. This study was approved by the Institutional Review Board of Assiut faculty of Medicine and performed in accordance with the principles of the Declaration of Helsinki.

Statistical Analysis

Data were verified, coded and analyzed using IBM-SPSS 21.0 (IBM-SPSS Inc., Chicago, IL, USA) *. Descriptive statistics: Means, standard deviations, medians, ranges and percentages were calculated. Test of significances: chi-square/Fisher's Exact test was used to compare the difference in distribution of frequencies among different groups. For continuous variables; independent t-test analysis was carried out to compare the means of normally distributed data. Kaplan–Meier curve was used to estimate the median survival time. The Log-rank test was used to compare survival curves between the categories of the explanatory variables. Multivariate Cox Hazard regression analysis was calculated to investigate the significant factors influencing recurrence free survival (RFS) and progression free survival (PFS) (Hazard Ratio, 95% confidence interval). ROC curve was depicted for the diagnostic performance of tumor depth of invasion, analyzed as area under the curve (AUC), standard error (SE) and 95% confidence interval (CI). Validity statistics: sensitivity, specificity, positive and negative predictive values (PPV and NPV) were calculated. A significant p value was considered when it is equal or less than (0.05).

Results

Patients' Characteristics

This study included 57 patients with pT1 classic urothelial carcinoma, their characteristics are displayed in Table 1. As regards tumor size, 37 (64.9%) patients had tumor more than 3 cm. and 23 (40.4%) of tumors were multifocal. Most tumors 43 (75.4%) had papillary architecture and 34 (59.6%) were high grade. Concomitant CIS was detected in 8 (14%) cases and LVI was found in 6 (10.5%) of our patients.

Assessment of muscularis mucosa invasion showed 28 (49.1%) substaged as A and 29 (50.9%) substaged as B. According to metric substaging system 16 (28.1%) were T1 m and 41 (71.9%) were T1e. According to ROL system, 22 (38.6%) were ROL1 and 35 (61.4%) were ROL2. As regards to depth of invasion 27 (47.4%) of our cases had invasion less than or equal to 1 mm and 30 (52.6%) cases had depth of invasion more than 1 mm. (Fig. 1) (Table 1).

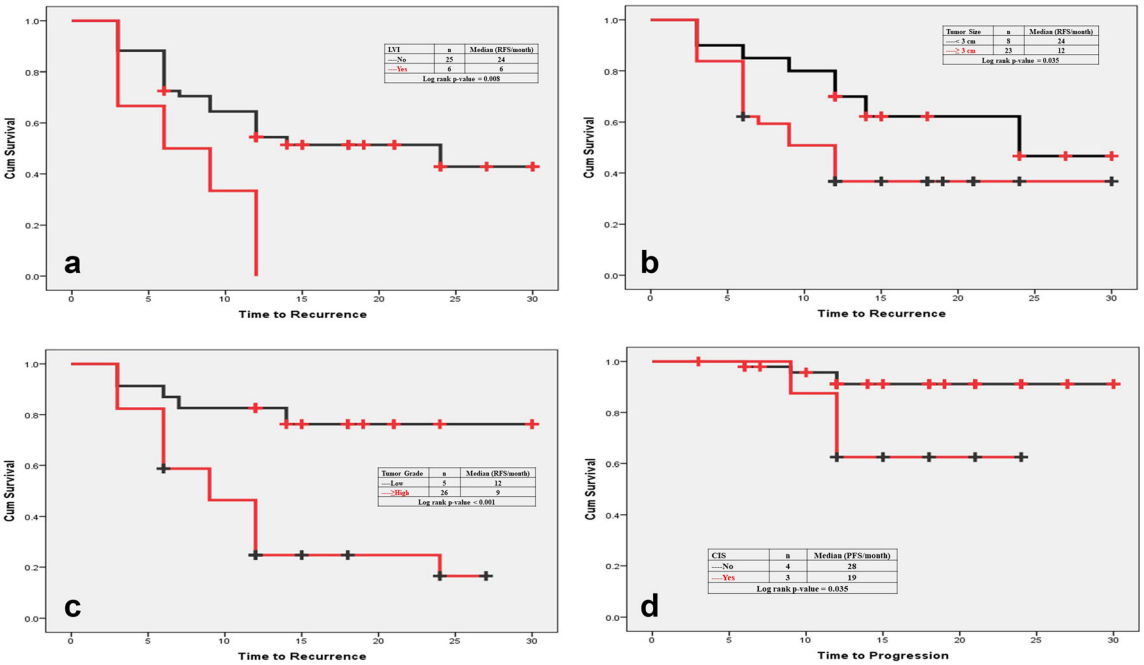

Fig. 3 **a** Kaplan-Meier survival plot of patient recurrence dependent on lymphovascular invasion ($p = 0.008$, log rank test). **b** Kaplan-Meier survival plot of patient recurrence dependent on tumour size ($p = 0.035$, log rank test). **c** Kaplan-Meier survival plot of patient recurrence dependent on tumour grade ($p < 0.001$, log rank test). **d** Kaplan-Meier survival plot of tumour progression dependent on concomittent CIS ($p = 0.035$, log rank test)

Recurrence

Of all patients, 31 (54.4%) had recurrence. There was significant association between tumor size, tumor architecture, LVI, tumor grade, and tumor recurrence. All 4 substaging systems applied also showed significant association with recurrence (Table 2).

On univariate regression analysis patients having, tumor size more than 3 cm, solid tumor architecture, high grade, substage B according to muscularis mucosa invasion, substage T1e according to metric substaging system, ROL 2 as regards ROL system and tumor depth more than 1 mm were associated with recurrence. On multivariate analysis all the four substaging systems, tumor size, grade and tumor type had significant prognostic value for recurrence (Table 4).

The Kaplan-Meier curves also illustrate the significant impact of the 4 substaging systems, LVI, size and tumor grade on recurrence (Figs. 2 and 3).

Progression

Of the total patients, 7 (12.3%) progressed. Tumor size, tumor architecture, associated CIS were significantly associated with progression. However, only the metric substaging method was associated with tumor progression (Table 3).

On univariate and multivariate regression analysis solid tumor architecture and CIS had significant prognostic value for tumor progression (Table 5). Only CIS had significant impact on progression by Kaplan-Meier log rank test (Fig. 3-d).

Tumor Depth as a Method for Substaging

The mean tumor depth for our cases was (1.31 ± 0.1 mm.), range (0.01–3.79 mm.). ROC analysis of the depth of invasion as indicator of muscularis mucosa invasion was performed. The overall accuracy as measured by the AUC was 0.764 and 1 mm was identified as the best cutoff. We compared 4 cutoff points (0.5, 1 mm, 1.5 and 3 mm) and found that 1 mm cutoff had higher accuracy (80.2%) than 0.5, 1.5 and 3 mm whose accuracy were (62.5%, 71% and 55%) respectively, and higher negative predictive value NPV (90.5%) compared to 0.5, 1.5 and 3 mm which had NPV (66.7, 63.3% and 52.6%) respectively (Fig. 4, Tables 6 and 7).

Discussion

NMIBC progressing to MIBC has an unfavorable prognosis even when treated with radical cystectomy hence there is an essential need for early detection of such cases to guide their timely management and decision planning [11]. Recently, the WHO and the eighth edition of AJCC cancer staging

Table 3 Clinical history and demographics by disease progression status

Parameter	Category	Non-progressor (no. = 50)	Progressor (no. = 7)	*P* value
Age/years	• Mean ± SD	64.14 ± 9.8	59.57 ± 10.4	= 0.313*
Sex	• Female	6	1	= 0.622**
	• Male	44	6	
Tumour Size	• < 3 cm	20	0	= **0.039****
	• ≥ 3 cm	30	7	
ROL System	• ROL1 ≤ 1 mm	21	1	= 0.161**
	• ROL2 > 1 mm	29	6	
Metric substaging	• ≤ 0.5 mm	16	0	= **0.044****
	• > 0.5 mm	34	7	
MM invasion Level	• A	26	2	= 0.226**
	• B	24	5	
Tumour Depth	• Mean ± SD	1.22 ± 0.1	1.98 ± 0.9	= 0.062*
Tumour Depth Category	• ≤ 1 mm	26	1	= 0.068**
	• > 1 mm	24	6	
Multiplicity	• Solitary	30	4	= 0.596**
	• Multiple	20	3	
Tumour Architecture	• Solid	10	4	= **0.033****
	• Papillary	40	3	
Tumour Grade	• Low	22	1	= 0.137**
	• High	28	6	
CIS	• Present	5	3	= **0.019****
LVI	• Present	4	2	= 0.097**

*T-test was used to compare the mean difference between groups

**Fisher's Exact test was used to compare the proportion difference between groups

recommended substaging T1 bladder cancer, although the optimal method is yet to be determined [12].

The most extensively studied method for T1 substaging is muscularis mucosa invasion. The limitations of this method include that muscularis mucosa is not always present and its presence in the biopsy range from 15%–83%. In its absence, the large vessels in the lamina propria could be used as a substitute based on the fact that they are closely related to MM. Also, as it is a discontinuous layer, the patient may have muscularis mucosa in some fragments, but those fragments are not the ones which showed tumor invasion [13].

Paner et al. stated that the ability of T1a/b substaging to predict outcome was 68% [14]. In our study, muscularis mucosa identification rate was 75.4% and we found significant association between MM invasion and tumor recurrence in univariate ($p < 0.001$) and multivariate analysis (HR = 4.284, 95% CI = 1.168–15.704), ($p = 0.028$) but not with tumor progression. Other studies reported insignificant difference in both recurrence and progression by MM invasion [15].

On the other hand millimetric depth of tumor invasion has been advocated by some studies as being more reproducible, objective and prognostically significant [7]. However, other

Table 4 Cox proportional hazard regression analysis for recurrence

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
ROL System (ROL2)	1.421	1.052–7.764	= **0.021**	3.644	1.485–8.941	= **0.005**
Metric substaging (> 0.5 mm)	2.854	1.398–12.060	= **0.006**	3.534	1.231–10.142	= **0.019**
Tumor Substage (B)	7.033	2.768–17.866	< **0.001**	4.284	1.168–15.704	= **0.028**
Tumor Depth in mm	5.112	2.145–12.181	< **0.001**	2.852	1.345–10.101	= **0.011**
Tumor Size (≥ 3 cm)	5.240	1.690–6.824	= **0.001**	2.050	1.190–4.618	= **0.031**
Tumor Grade (High)	1.557	1.018–6.947	= **0.031**	4.869	1.855–12.782	= **0.001**
Tumor Architecture (Solid)	4.002	1.899–8.435	< **0.001**	3.661	1.578–8.497	= **0.003**
CIS	1.667	0.605–4.591	= 0.323			
LVI	1.292	0.454–3.682	= 0.231			

HR Hazard Ratio, *CI* Confidence Interval

Table 5 Cox proportional hazard regression analysis for progression

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
ROL System (ROL2)	4.382	0.527–36.407	= 0.171			
Metric substaging (> 0.5 mm)	6.771	0.045–40.702	= 0.279			
Tumor Substage (B)	2.804	0.544–14.456	= 0.218			
Tumor Depth in mm	6.522	0.785–54.189	= 0.083			
Tumor Size (≥ 3 cm)	13.342	0.824–45.682	= 0.274			
Tumor Grade (High)	4.426	0.533–36.763	= 0.169			
Tumor Architecture (Solid)	5.015	1.122–22.420	= **0.031**	4.049	1.122–12.650	= **0.035**
CIS	4.389	1.182–9.616	= **0.035**	3.054	1.062–14.469	= **0.021**
LVI	2.984	0.579–15.389	= 0.191			

HR Hazard Ratio, *CI* Confidence Interval

studies stated that measuring the depth of invasion is not straight forward especially in badly oriented specimens. Also, an overlying epithelium needs to be present for accurate measurement [13]. In this regards, it is interesting to state that in the original study done by cheng et al. [7], the depth of invasion was measured even in absence of overlying mucosa or in badly oriented biopsies by measuring the shortest distance of invasive focus to avoid overestimation of the depth. By doing this in the current study, this measurement was possible in all cases and we found association with tumor recurrence in univariate ($p < 0.001$) and multivariate analysis (HR = 2.852, 95%CI = 1.345–10.101), ($p = 0.011$).

As regards the depth of invasion cutoff value, cheng et al. [7] suggested that the depth of invasion could be prognosticator for T1 tumor progression and although the results were significant with cut off of invasion ≥1.5 mm. This has not been validated in other studies [3, 13] except one study which confirmed the presence of strong correlation between tumor depth and progression but they proposed a different cut off value of 3 mm [13]. Because the reported mean thickness of the lamina propria is 1.4 mm which is smaller than both previous cut off values, these cut off values have not been validated [16]. When we applied ROC curve to our data we found that using 1 mm cut off yields higher accuracy than previously tested cut off values (1.5 mm and 3 mm) and also was better than using 0.5 mm as a cut off value. So we advocate the validation of 1 mm cut off in future studies.

Tumor diameter measurements (metric substaging and ROL system) have been studied in the last few years as a reproducible and user friendly way to substage T1 BC. Moreover they are not affected by tissue orientation and don't need an overlying epithelium [13]. It was first described by van der Aa et al., [9] with diameter 0.5 mm (one high power field) to substage T1 BC into T1 m and T1e. It was proposed that tumor with larger diameter of invasion are more likely to extend below MM and have greater depth of invasion [12]. In agreement with previous studies we found that the application of this system was possible in 100% of cases and it showed to be the only substaging method associated with tumor recurrence and progression ($p = 0.005$ & 0.044 respectively), however it correlated only with recurrence in univariate and multivariate analysis. Using the metric substaging system some studies failed to detect a prognostic significance [17]. However in other studies a significant correlation was found in univariate and multivariate analysis [9, 10, 13, 18, 19].

Table 6 Diagnostic performance of Tumour depth in mm, analysed as area under the curve (AUC) (95% CI)

	Goodness criteria Tumour Depth in mm			
• AUC	0.764			
• Cut-off	0.5	1.0	1.5	3
• Accuracy	62.5%	80.2%	71%	55%
• Sensitivity, %	75%	67.5%	42%	10%
• Specificity, %	50%	93%	100%	100%
• PPV, %	60%	74.1%	100%	100%
• NPV, %	66.7%	90.5%	63.3%	52.6%

*Sensitivity (true positives/all diseased); specificity (true negatives/all non-diseased);

PPV (true positives/all test positives); NPV (true negatives/all test negatives)

Table 7 Diagnostic performance of different cut-offs of tumour depth as Indicator of MM involvement, analysed as area under the curve (95% CI)

	AUC*	95% CI+	SE**	*P* value***
• 0.5 mm	0.622	0.446–0.798	0.090	0.173
• 1 mm	0.801	0.679–0.924	0.062	= 0.001
• 1.5 mm	0.791	0.677–0.904	0.058	= 0.001
• 3 mm	0.547	0.397–0.417	0.085	0.604

**AUC* Area under the Curve, ***SE* Standard Error, +*CI* Confidence Interval

***Null hypothesis: true area = 0.5

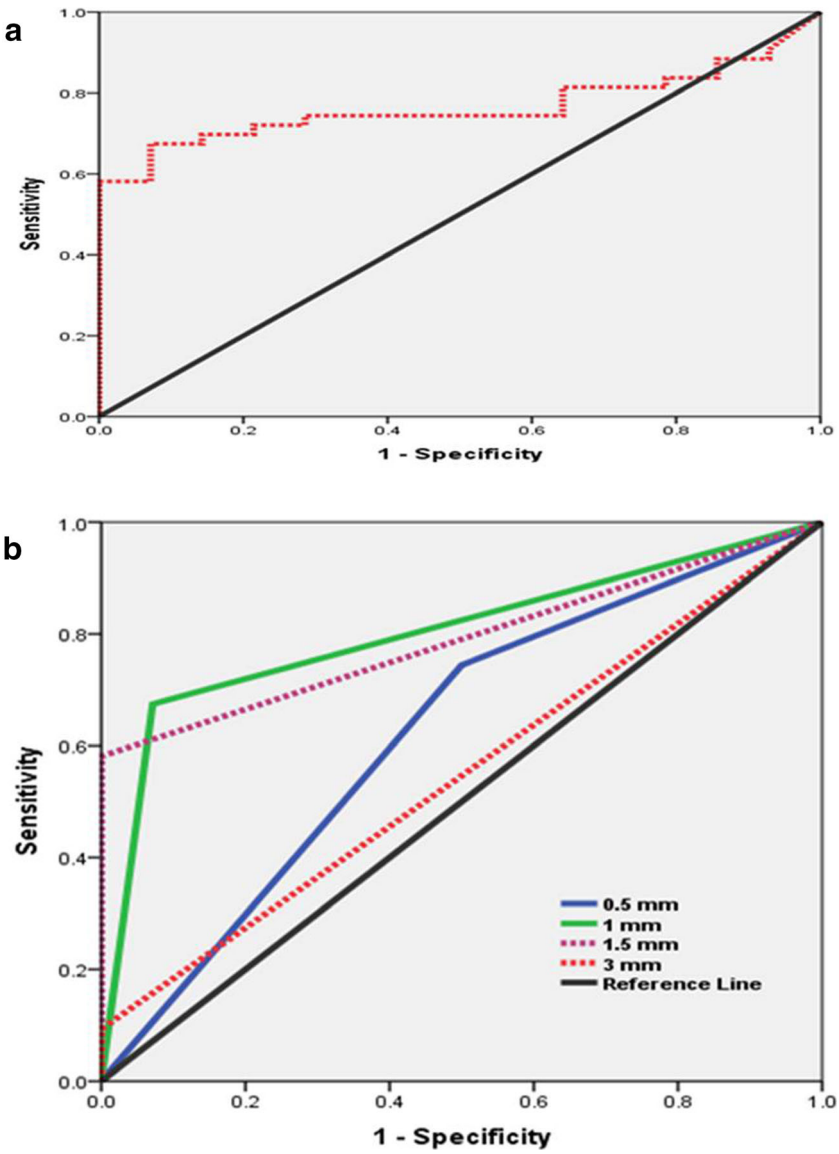

Fig. 4 **a** ROC curve for tumor depth as indicator of MM involvement. **b** ROC curve for different cut-offs of tumour depth as indicator of MM involvement

When we applied ROL system with cut off 1 mm (using 20× lens) we also found a significant association and correlation with tumor recurrence in univariate ($p = 0.021$) and multivariate analysis (HR = 3.644, 95% CI 1.485–8.941) (p = 0.005). Based on our findings, using the ROL system and increasing the cutoff value to 1 mm didn't show more prognostic significance.

Several studies confirmed that CIS, tumor size [9, 19–21] and grade [3, 16, 22] are important prognostic factors for T1 BC. In our study CIS and a solid growth pattern were significantly correlated with tumor progression. Tumor size ≥3 and high grade tumors were prognostic factors for recurrence.

Regarding LVI, Martin-Doyle et al. [21] found it to be a prognostic factor for recurrence but not for progression. Similarly other studies [13, 23] reported that LVI was associated with worse outcome of patients. In the present study, we found association of LVI with tumour recurrence but not with progression and this may be due to the relatively low number of cases. Larger study may give reliable data on this parameter.

The limitations of this study are the relatively short follow up period and relatively few number of cases.

Conclusion

In view of our results using 1 mm cutoff in measuring the depth and 5 mm for the diameter of infiltration may provide clinically relevant information to guide a more personalized therapy.

We recommend establishment of pathological risk stratification for NMIBC patients and suggest tumor depth of 1 mm and 0.5 mm diameter of invasion as items in the pathology report together with other prognosticators as CIS, tumor

architecture, grade, size and LVI with possible evolution of a scoring system in the future.

Compliance with Ethical Standards

Conflict of Interest Authors declare no conflict of interest.

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