

ORIGINAL ARTICLE

Proliferation markers and DNA content analysis in urinary bladder TaT1 urothelial cell carcinomas: identification of subgroups with low and high stage progression risks

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Aims: To evaluate whether in situ biomarkers Ki67, mitotic activity index (MAI), p53, mean area of the 10 largest nuclei (MNA10), and whole genome DNA ploidy by flow and image cytometry (FCM and ICM, respectively) have independent prognostic value in urinary bladder urothelial cell carcinomas (UCs).

Methods: Ki67 and p53 immunoquantitation was performed in TaT1 consensus diagnosis UCs. MAI and MNA10 were also determined. Single cell suspensions were stained (DAPI for FCM; Feulgen for ICM). There was enough material for all measurements in 171 cases. Kaplan-Meier curves and multi-variate survival analysis (Cox) were used to assess the prognostic value of all features (including classic clinicopathological risk factors, such as stage, grade, multicentricity, carcinoma in situ).

Results: Thirteen (7.6%) patients progressed. Of the classic factors, grade was strongly prognostic in univariate analysis, as were all the biomarkers. In multivariate analysis, the strongest independent combinations for progression were MNA10 (threshold (T) = 170.0 μm^2) plus MAI (T = 30), or MNA10 (T = 170.0 μm^2) plus Ki67 (T = 25.0%). p53 (T = 35.2%) plus Ki67 (T = 25.0%) also predicted progression well, with high hazard ratios, but p53 measurements were not as reproducible as the other features. The prognostic value of the quantitative biomarkers exceeded that of the classic risk factors and DNA ploidy. The sensitivity, specificity, positive, and negative predictive values of MNA10/MAI or MNA10/Ki67 at the thresholds mentioned were 100%, 79%, 57%, and 100%, respectively. These feature combinations were also strongest prognostically in the high risk treatment subgroup.

Conclusions: The combined biomarkers MNA10/Ki67 or MNA10/MAI are more accurate and reproducible predictors of stage progression in TaT1 UCs than classic prognostic risk factors and DNA ploidy.

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At recurrence, 5–15% of TaT1 urothelial cell carcinomas (UCs) of the urinary bladder show stage progression. This is associated with higher grades,^{1–3} but the inter-observer and intra-observer variations of grade may be as high as 15–40%.^{4–6} Recently, it was found that the evaluation of the same specimens by different pathologists could result in grade and stage differences, which would have enormous therapeutic implications.⁷ Thus, more objective prognosticators have been sought. Popov *et al* found that a combination of MIB-1 and p53 was a stronger prognosticator of stage progression than the individual variables.⁸ However, sampling and thresholds of quantitative features vary,⁹ which makes the comparison of results difficult. Therefore, the need for objective and reproducible prognosticators remains high. We previously studied the prognostic value of proliferation associated biomarkers Ki67 and mitotic activity index (MAI), together with p53 and mean area of the 10 largest nuclei (MNA10), using specialised computerised morphometric in situ technology with systematic random sampling, in a large group of patients with TaT1 UCs. These features are highly reproducible and the “URO-QP model” using these quantitative biomarkers can accurately identify UCs at high risk for progression, both in low and high risk treatment groups (as defined by classic prognostic factors such as stage, grade, carcinoma in situ, and multicentricity). Many other studies have also found that DNA ploidy correlates with grade, lamina propria invasion, and prognosis.^{10–13} Flow cytometry (FCM) has been used mostly for ploidy measurements. However, FCM has the

disadvantage of measuring mixtures of cells, including non-tumour cells, so that other studies have used image cytometry (ICM),^{14–15} which has the definitive advantage of measuring tumour cells only. However, the ICM technology used in these studies was slow and had a high coefficient of variation (CV), so that the usefulness of this technique for daily practice has been questioned.¹⁶ Recently, we have described a fully automated ICM method that measures thousands of cells (mainly tumour cells) in minutes, with a low CV. This method is also highly accurate at predicting stage progression, more so than FCM.¹⁷ In our present study, we have evaluated the additional value of DNA FCM and ICM over the URO-QP model.

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Abbreviations: CV, coefficient of variation; DI, DNA index; FCM, flow cytometry; FOV, field of vision; H&E, haematoxylin and eosin; HR, hazards ratio; ICM, image cytometry; IHC, immunohistochemistry; MAI, mitotic activity index; MNA10, mean area of the 10 largest nuclei; SPF, S phase fraction; T, threshold; UC, urothelial cell carcinoma; WHO, World Health Organisation

Table 1 Results of progression free survival analysis of urinary bladder tumours, TaT1 urothelial cell carcinomas

Variable	N	Progressed	Censored	% Progression free survival	Log rank	p Value	HR	95% CI
<i>Models in the total group (n=171)</i>								
Sex								
Male	133	8	125	93.9	1.9	0.16	2.3	0.7 to 6.6
Female	38	5	33	86.4				
Age								
<60	47	2	45	95.7	1.9	0.60	1.0	–
60–67	45	3	42	93.3			1.6	0.3 to 9.9
67–76	42	5	37	88.1			2.8	0.6 to 14.9
>76	37	3	34	91.9			1.9	0.3 to 11.1
TaT1								
Ta	132	8	124	93.9	2.0	0.15	2.2	0.7 to 6.7
T1	39	5	34	87.2				
Multicentricity								
Yes	19	2	17	89.5	0.4	0.55	0.6	0.1 to 2.9
No	152	11	141	92.5				
Carcinoma in situ								
Yes	4	1	3	75.0	1.7	0.19	0.3	0.0 to 2.2
No	167	12	155	92.8				
Original grade								
1	38	1	37	97.4	14.1	0.0009	1.0	–
2	78	2	76	97.4			1.0	0.9 to 11.0
3	55	10	45	81.8			8.0	1.0 to 62.6
Revised grade								
1	40	0	40	100.0	11.8	0.0026	1.0	–
2	69	3	66	95.6			Infinite	Infinite
3	62	10	52	83.3			Infinite	Infinite
Treatment								
Low risk	28	0	28	100.0	17.4	0.0002	1.0	–
Intermediate	75	1	74	98.7			Infinite	Infinite
High risk	68	12	56	82.4			Infinite	Infinite
Flow cytometry								
DI = 1.00	102	2	100	98.0	13.6	0.0002	10.1	2.2 to 45.9
All others	69	11	58	84.1				
DI <1.3	113	3	110	97.4	13.6	0.0002	7.9	2.2 to 28.9
All others	58	10	48	82.8				
Image cytometry								
DI = 1.00	104	2	102	98.1	13.8	0.0002	10.2	2.2 to 46.0
All others	67	11	56	83.6				
DI <1.3	109	2	107	98.2	16.1	0.0001	11.6	2.6 to 52.5
All others	62	11	51	82.3				
DI >1.3 and SPF >16.2	33	8	25	75.8	16.8	0.0000	7.7	2.3 to 22.4
All others	138	5	133	96.4				
MNA10								
<90.0	41	0	41	100.0	36.9	0.0000	1.0	–
90.0–119.0	42	0	42	100.0			1.0	Infinite
119.1–179.0	38	0	38	100.0			1.0	Infinite
>179.0	50	13	37	74.0			375.0	Infinite
<170.0	121	0	121	100.0	36.86	0.0000	372.8	Infinite
>170.0	50	13	37	74.0				
MAI								
<2	48	0	48	100.0	48.9	0.0000	1.0	–
2–10	35	0	35	100.0			1.0	Infinite
10–30	46	0	46	100.0			1.0	Infinite
>30	42	13	29	69.1			916.7	Infinite
≤30	129	0	129	100.0	48.9	0.0000	909.5	Infinite
>30	42	13	29	69.0				
Ki67 area%								
<5.0	44	0	44	100.0	45.9	0.0000	1.0	–
5.0–13.0	44	0	44	100.0			1.0	Infinite
13.0–26.0	39	0	39	100.0			1.0	Infinite
>26.0	44	13	31	70.45			719.0	Infinite
≤25.0	127	0	127	100.0	45.9	0.0000	712.3	Infinite
>25.0	44	13	31	70.45				
p53 area%								
<8.2	39	0	39	100.0	26.6	0.0000	1.0	–
8.2–17.1	44	0	44	100.0			1.0	Infinite
17.1–36.0	42	2	40	95.2			Infinite	Infinite
>36.0	46	11	35	76.1			Infinite	Infinite
MNA10 (170) and Ki67 (25)								
All others	135	0	135	100.0	60.8	0.0000	2216.5	Infinite
>170.0 >25.0	36	13	23	63.9				

Table 1 continued

Variable	N	Progressed	Censored	% Progression free survival	Log rank	p Value	HR	95% CI
MNA10 (170) and MAI (30)								
All others	138	0	138	100.0	69.3	0.0000	4215.3	Infinite
>170.0 >30	33	13	20	60.6				
Ki67 (25) and p53 (35.2)								
All others	141	2	139	98.6	52.7	0.0000	33.9	Infinite
>25.0 >35.2	30	11	19	63.3				
<i>Models within the BCG treated high risk subgroup (n=68)</i>								
MNA10 (170) MAI (30) low	36	0	36	100.0	16.6	0.0000	77.8	Infinite
MNA10 (170) MAI (30) high	32	12	20	62.5				
MNA10 (170) Ki67 (25) low	34	0	34	100.0	14.3	0.0000	95.4	Infinite
MNA10 (170) Ki67 (25) high	34	12	22	64.7				

Line 1, 1st figure: subgroup 1 reference value; 2nd figure: subgroup 2 v 1; 3rd figure: subgroup 3 v 1; 4th figure: subgroup 4 v 1.
CI, confidence interval; DI, DNA index; HR, hazards ratio; N, number; MAI, mitotic activity index; MNA10, mean area of the 10 largest nuclei; SPF, S phase fraction.

MATERIALS AND METHODS

Tissues from 191 primary UCs, diagnosed from 1995 to 1997, were obtained by transurethral resection or biopsy. Mean (SD) age at the time of diagnosis was 66.9 (39–92) years; all patients were white. Tissues were fixed in 4% formaldehyde, dehydrated, paraffin wax embedded, and haematoxylin and eosin (H&E) stained 4 µm thick sections were made. The worst differentiated area (measurement area: minimally 2 × 2 mm and maximally 5 × 5 mm) was carefully demarcated for Ki67, MAI, p53, and MNA10 measurements, avoiding tangentially cut, necrotic, damaged, or inflamed parts. Staging and grading according to the Union Internationale Contre le Cancer (UICC)¹⁸ and World Health Organisation (WHO) 1999¹⁹ criteria was carried out by three independent experienced pathologists (at the time of performing the study, the WHO2002 was not yet available and therefore could not be used). Sections were graded in the worst differentiated area. In T1 cases, this is nearly always in the infiltrating component. The difference between the WHO99 and WHO73 system is mainly in grade 1 cases, and the overall difference is small. The main interest in our present evaluation was in the prognostic differences within the higher grade cases (which are therapeutically more important). This means that, in practice, the grading system used was very similar to the WHO73 classification. Three review rounds were organised to obtain consensus diagnosis. Patients were regarded as low, intermediate, or high risk on the basis of Ta or T1 stage, grade, multicentricity, and carcinoma in situ component.^{2,20,21} Follow up and postoperative treatment depended on this risk scheme.⁹ Follow up data were retrieved from medical records and the computerised national pathology archive. All patients were followed until progression or until the end of the follow up period; none was lost to follow up.

Immunohistochemistry and quantitative image analysis

The methods used for immunohistochemistry (IHC) and quantitative image analysis have been described in detail before.²² For IHC, 4 µm thick paraffin wax embedded sections, adjacent to the H&E sections used for grade, MAI, and MNA10 assessment, were used. The presence and location of the TaT1 lesion was carefully controlled (“sandwich technique”) to guarantee that the Ki67 and p53 sections contained the same lesion.

Ki67 immunohistochemistry was performed with the QPRODIGIT 6.1 image analysis system (Leica, Cambridge, UK), at a final magnification of ×600 (objective, ×40; numerical aperture, 0.75), using motorised scanning stage random sampling. In each field of vision (FOV), the same four endpoints of an electronic grid are used to register Ki67 negative/positive nuclei (which were thus randomly selected within the

measurement area). At least 150 nuclei were counted in this way (average, 236/case). Thus, the test grid sample point could be Ki67 positive, negative, or neither of the two. Ki67 percentage (and in the same way also p53 percentage) was defined as ((Ki67 positive)/(Ki67 positive + Ki67 negative)) × 100. Computerised morphometric MNA10 analysis was performed as follows. First, the observer systematically “scans” the measurement area, consisting of maximally 5 × 5 mm of tumour tissue. Thus, maximally, 30–40 FOVs (using the ×20 lens) are scanned. Subsequently, the observer zooms in (using the ×100 lens) on the subjectively largest nucleus in each FOV and measures the profile of the monitor projected image (total magnification, ×1400). Of the 30–40 nuclear profiles, the mean of the largest 10 is calculated (MNA10).

MAI was assessed by counting mitotic figures in the measurement area in 10 consecutive FOVs at a final magnification of ×400 (objective, ×40; numerical aperture, 0.75; diameter at specimen level, 450 µm²; total area at specimen level of 10 FOVs, 1.5896 mm²). A FOV was only accepted if a minimum of 75% consisted of urothelial tumour cells and stroma. Mitotic figures were counted using well established criteria.⁵

FCM and ICM

Methods for FCM and ICM have been described in detail before¹⁷ and will be summarised here. All samples were analysed within three hours of staining, on a commercial flow cytometer (PARTEC, Munster, Germany). The CV was defined as the ratio of the half width at 61% (2SD) of the G0/G1 peak on the abscissa. Trout erythrocytes served as external control cells. The fully automated DNA image cytometry method uses densitometric and geometric filters carefully fine tuned for UCs. It has a low CV and the overall measuring time is less than five minutes for each sample of 3000 cells or more.

In the histograms, the 2c peak was identified interactively, after which the histogram was scaled with a fixed number of 256 bins to obtain standardised histograms for all samples. After the measurements, the histograms were filed in ASCII format and analysed with the MultiCycle computer program (Phoenix Flow Systems, San Diego, USA). The DNA index (DI) was determined as the ratio of the second G0/G1 to the first G0/G1 peak in the DNA histogram, assuming that the first G0/G1 peak to the left belonged to the diploid cells, either from tumour cells or from other cells in the specimen. A diploid (2c) tumour showed only the G0/G1 peak in the expected histogram region. Diploid tumours were defined as DI = 1.00. An aneuploid tumour showed an additional peak to the 2c peak. Near diploid/aneuploid tumours were defined as 1.00 < DI < 1.30. Low aneuploid tumours had 1.30 < DI < 1.90. Tetraploid tumours showed a G0/G1 peak on a double distance of the diploid peak with 1.90 < DI < 2.10. The 4c peak should have a height of at least

Table 2 Correlation (Spearman's test) between the biomarkers and DNA analysis results

		MNA10	Ki67	MAI	p53	DI FCM	SPF FCM	DI ICM	SPF ICM
MNA10	R	1.00	0.74	0.67	0.71	0.62	0.40	0.60	0.56
	P		0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ki67	R		1.00	0.82	0.69	0.53	0.45	0.55	0.56
	P			0.000	0.000	0.000	0.000	0.000	0.000
MAI	R			1.00	0.62	0.53	0.40	0.55	0.51
	P				0.000	0.000	0.000	0.000	0.000
p53	R				1.00	0.46	0.33	0.44	0.47
	P					0.000	0.000	0.000	0.000
DI FCM	R					1.00	0.57	0.79	0.41
	P						0.000	0.000	0.000
SPF FCM	R						1.00	0.45	0.37
	P							0.000	0.000
DI ICM	R							1.00	0.50
	P								0.000
SPF ICM	R								1.00
	P								

Note the strong correlation ($R > 0.65$, bold) for the in situ biomarkers with each other, but not with the DNA cytometric features (either by ICM or FCM). DI, DNA index; FCM, flow cytometry; ICM, image cytometry; MAI, mitotic activity index; MNA10, mean area of the 10 largest nuclei; P, probability of no significance (< 0.05); R, correlation coefficient; SPF, S phase fraction.

10% of the diploid peak for classification as tetraploid. High aneuploid tumours were defined as $DI > 2.10$. The CV of the diploid peak was defined as the ratio of the half width at 61% (2SD) of the Go/G1 peak on the abscissa.

Statistics

SPSS 10.0 for Windows (SPSS, Chicago, USA) was used. Progression in recurrent UCs was defined as microscopic proof of a higher stage than in the primary tumour. Progression free survival was taken as the primary diagnosis date until the occurrence of histological progression or recurrence. Patients alive without recurrence were censored at the last follow up date. Variables in the analysis were sex, age, stage, original grade, revised grade, carcinoma in situ component, multicentricity, treatment, DNA ploidy of FCM and ICM, DNA ploidy into five groups (see above), S phase fraction (SPF) for FCM and ICM, Ki67, MAI, p53, and MNA10. Survival analysis was performed (Kaplan-Meier) and continuous variables were divided into two, three, or four groups of the same size using thresholds of the median, tertiles, or quartiles (for categorisation see table 1). If survival results of adjacent groups did not differ, they were combined (which resulted in one threshold, which always was the 75th centile). The hazard ratios (HRs) were calculated (HRs in subgroups without progression could sometimes not be calculated or resulted in infinite values). Multivariate analysis was performed (Cox) to find the best prognostic combination of clinicopathological and quantitative features, leaving out strongly correlated and non-significant features. In cases with equally predictive features, well reproducible features were given priority. Furthermore, previously described thresholds from the URO-QP model were analysed.

RESULTS

Thirteen (7.6%) of the 171 patients progressed. The median follow up time in the non-progression and progression groups was 51.1 months (range, 31.0–69.6) and 13.4 months (range, 2.2–31.4), respectively. Table 2 shows the correlation of the in situ biomarkers, flow and image cytometric features. Note that MNA10 correlates well with all the proliferation markers and with the DI of FCM and ICM. The correlation with SPF by ICM was lowest. Similarly, MAI, Ki67, and SPF ICM correlated well, but the correlation with SPF FCM is weakest. Figure 1 shows that Ki67 and p53 correlate strongly ($r = 0.69$; $p < 0.0001$) and when combined they predict progression well. However, the prognostic information mainly resides in Ki67 (which is also more reproducible than p53).

Table 1 shows the single variate progression free survival results. Most features are significant, although in general the HRs of the quantitative in situ biomarkers are higher than those of stage, grade, and other clinicopathological variables. Figure 2 shows the best predicting set of features for ICM (which predicted progression more accurately than FCM).¹⁷ The essential prognostic threshold of the in situ biomarkers was consistently below or above the 75th centile, as described before for the URO-QP model in another patient group. In particular, Ki67 (threshold (T) = 25.0%), MAI (T = 30), and MNA10 (T = 170.0 μm^2) performed very well. MNA10 (T = 170.0 μm^2) and MAI (T = 30) was the strongest prognostic combination, but MNA10 (T = 170.0 μm^2) and Ki67 (T = 25.0%) predicted progression almost as well (Cox regression). The sensitivity, specificity, positive, and negative predictive values of MNA10 and MAI were 100%, 79%, 57%, and 100%, respectively, and these figures were almost the same for MNA10 and Ki67. The quantitative variables were also prognostic in the high risk treatment subgroup. Postbiopsy treatment did not have independent additional prognostic value once the URO-QP progression model was taken into account. Figure 3 shows a scatter plot of MNA10

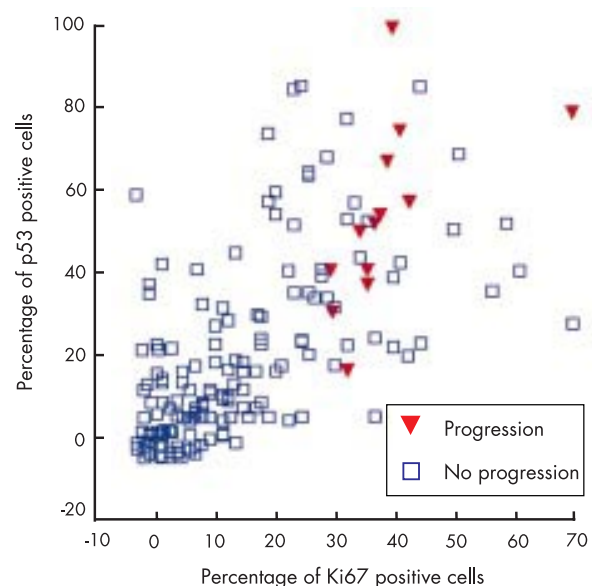


Figure 1 Percentages of p53 and Ki67 positive cells as predictors of progression in urinary bladder TaT1 urothelial cell carcinomas.

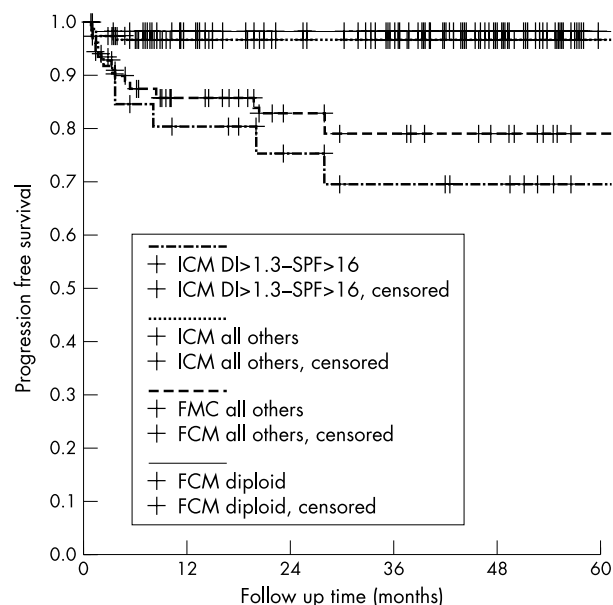


Figure 2 The best predicting set of features for image cytometry (ICM), which predicted progression more accurately than flow cytometry (FCM).

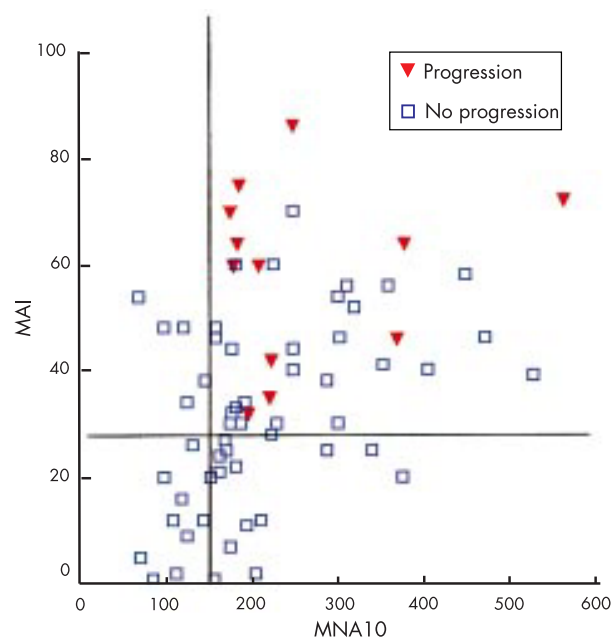


Figure 3 Mitotic activity index (MAI) and mean area of the 10 largest nuclei (MNA10) as predictors of progression in patients with high risk urinary bladder (mainly T1G3) urothelial cell carcinomas.

and MAI in the high risk treatment patients (mainly T1G3 patients). Patients with $MNA10 > 170.0 \mu m^2$ and $MAI > 30$ had a high progression risk (39.4%), contrasting with patients with one of these variables below the thresholds mentioned (0%). The analysis of other thresholds of MNA10 and MAI in this subgroup did not improve this result. Figure 4 shows the URO-QP progression model for MNA10-Ki67 used in our laboratory.

DISCUSSION

Our results show that the URO-QP model—consisting of the in situ biomarkers Ki67, MAI, and MNA10—provides stronger

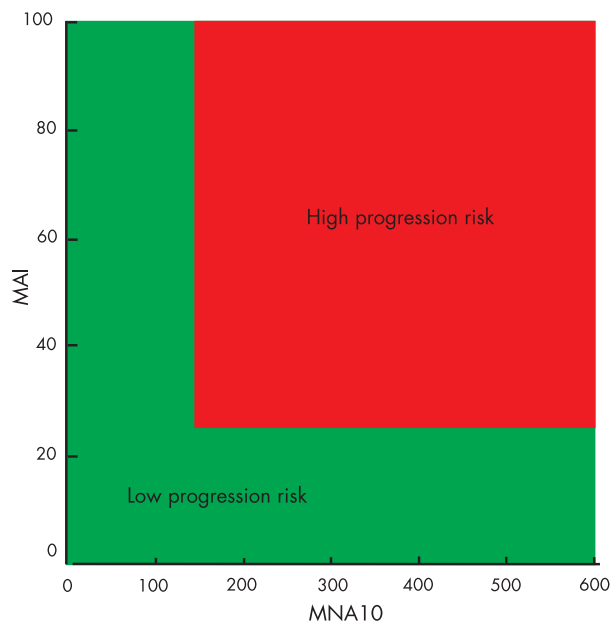


Figure 4 The URO-QP progression model for MNA10 (mean area of the 10 largest nuclei)/Ki67 used in our laboratory. MAI, mitotic activity index.

prognosticators than classic clinicopathological risk factors in TaT1 urothelial cell carcinomas. This biomarker combination is biologically interesting because it reflects the phenotypic expression of malignancy, is well reproducible, and is easily applicable in routine practice. Ki67 and MAI are measures of proliferation, which is one of the four important malignant phenotype criteria. Proliferation is of strong prognostic value in tumours of many different organs.^{23–24} Thus, it is not surprising that this also holds for UCs, as has been reported earlier.^{25–26} With regard to MNA10, we previously hypothesised that high MNA10 values could be either a reflection of aneuploidy or genomic instability. Our current results indicate that MNA10 is indeed correlated with DNA index (aneuploidy) (table 2) (Spearman coefficient, 0.62 and 0.60). It may be that MNA10 is also an expression of genomic instability, although other explanations should be considered. The subgroup of cells with the 10 largest nuclei within the worst differentiated area of a UC may well be an especially aggressive cell clone. Intratumour heterogeneity of cell clones with varying malignancy is a recognised phenomenon.²⁷ The advantage of computerised in situ morphometry of MNA10 is that malignant cell clones can be identified and selectively measured, strengthening the prognostic value.

Ki67 is positive in the G1, S, G2, and M phases of the cell cycle,²⁸ whereas MAI is a late M phase marker only. However, both proliferation biomarkers were strongly prognostic, confirming the results of others.^{29–30} In a previous study, we found that the decision threshold of MAI and Ki67 could be varied considerably without losing prognostic value.⁹ This confirms the strong correlation with the progression capacity of invasive UCs.

“The combination of MNA10/MAI or MNA10/Ki67 offers a solid basis for predicting the clinical outcome of TaT1 urinary bladder urothelial cell carcinomas”

p53 plays an important part in carcinogenesis, has a central role in cell cycle control, and acts as a checkpoint for DNA quality. In the case of DNA damage, p53 mediates the increased expression of p21, a cyclin dependent kinase inhibitor, which inhibits the cyclin dependent kinase–cyclin pathway,

Take home messages

- The combined biomarkers—mean area of the 10 largest nuclei (MNA10)/Ki67 or MNA10/MAI (mitotic activity index)—are more accurate and reproducible predictors of stage progression in TaT1 urothelial cell carcinomas than classic prognostic risk factors and DNA ploidy
- The p53/Ki67 combination also predicted progression well, with high hazard ratios, but p53 measurements were not as reproducible as the other features
- These feature combinations were also strongest prognostically in the high risk treatment subgroup

thereby blocking progress through the cell cycle. Thus, the prognostic role of p53 is understandable at the molecular biological level. However, staining for p53 is not as distinctive as that seen for Ki67, thereby reducing its reproducibility.³¹ Although p53 and Ki67 provide a prognostically important combination, most of the information resides in Ki67. Whatever the molecular biological basis for this, the high prognostic significance of proliferation associated features and MNA10 is a sign that our current findings are robust, and that these tests will be useful in future patients.

The reproducibility of quantitative features is another advantage. It is important that the quantitative biomarker combinations exceed the prognostic value of classic features. Thus, the combination of MNA10/MAI or MNA10/Ki67 offers a solid basis for predicting the clinical outcome of TaT1 urinary bladder UCs. A major advantage of the current methods is that they are independent of stage and grade, the assessment of which is not always reproducible.⁶ In contrast, the biological prognosticators described here are extremely reproducible, and our data confirm earlier results on other patient sets. The robustness, reproducibility, and easy applicability of the quantitative biomarkers are an important consideration for their implementation in daily routine use. To increase confidence even further, DNA ICM can be used as an additional quality control method for the other quantitative assessments. It is also important that the biological stage progression indicators provided accurate prognostic information in the high risk subset of T1G3 patients (fig 3). It might be useful to perform more frequent follow up biopsies as first choice in these patients to provide more information on data on quantitative features in this subset of patients. Although our current results have provided useful data, to assess the clinical robustness of a laboratory test it is also important to evaluate the method in independent test sets, preferably prospectively.³² Unfortunately, such studies are only rarely carried out. Two such evaluation studies (one retrospective, one prospective) are currently in progress.

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