

# Quantitative molecular grading of bladder tumours: A tool for objective assessment of the biological potential of urothelial neoplasias

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**Abstract.** The present study aimed to assess whether patients with bladder urothelial tumours can be more objectively stratified into low- and high-risk groups for recurrence and progression using a 2-tiered molecular grading scheme, than by subjective histopathological grading alone. Biopsy material from 45 consecutive patients with urothelial bladder neoplasias (2 papillary urothelial neoplasm of low malignant potentials, 18 pTa, 1 pTis, 19 pT1 and 5 pT2) was analysed for immunohistochemical Ki-67 and p53 expression. Labelling indices were assessed by automated cellular image analysis. UroVysion FISH test results were evaluated by automated signal counting, and DNA ploidy of single nuclei preparations were measured by image cytometry. Sixty-nine percent of cases showed >10% Ki-67 LI, 64% had >10% p53 LI, 53% revealed DNA aneuploidy and 56% expressed a high-risk FISH pattern. Based on a combination of single molecular markers, 75% of neoplasias were classified as high molecular grade. Tumour stage and histopathological grade were significantly associated with FISH pattern, DNA ploidy and MIB1 LI. Stage was also related with molecular grade. Clinical outcome showed a significant correlation with MIB1 LI and molecular grade. P53 had neither diagnostic nor prognostic relevance, nor was there any correlation between histological and molecular grade. Our preliminary data strongly suggest that the combination of quantitative biomarkers provides superior and objective prognostic tools in bladder urothelial neoplasias compared to classic clinicopathological features and indices.

## Introduction

Most malignant neoplasms of the urinary bladder are urothelial carcinomas (UC), which frequently occur multifocally and have various clinical manifestations with different prognostic and therapeutic implications. Clinical diversity is associated with pathological and molecular dichotomy. Low-grade, superficial bladder cancers have a propensity to recur but are at minimal risk for progression and tumour-related death. In contrast, high-grade, muscle-invasive cancers often metastasise, resulting in poor clinical outcome (1). Clinical and pathological prognostic factors have been studied extensively (2,3). Despite well-recognized limitations of pathological assessment (4), stage and grade remain the standard estimates for outcome prediction. Recently, the European Organization for Research and Treatment of Cancer (EORTC) developed risk tables based on the combination of six routinely assessed factors: prior recurrence rate, number of tumours, tumour size, pT stage, grade and concomitant carcinoma *in situ* to predict the probability of recurrence and progression in individual patients (5).

However, the prognosis of patients, particularly of those labelled with 'superficial' bladder cancer is greatly variable. Therefore, there is a need to develop objective markers that can augment morphological risk assessment and assist therapy decisions.

Several biomarkers have been investigated for their role in the development and progression mechanisms of bladder carcinoma. Oncogenes, tumour suppressor genes and associated cell-cycle proteins, growth factors, cell adhesion molecules and chromosomal alterations have been identified in UC (6,7). Some of them hold considerable promise in the assessment of tumour aggressiveness [e.g. p53 (8), Ki-67/MIB1 (9,10) and DNA ploidy (11,12)]. However, only a few studies have analysed the validity of multiple molecular markers, which revealed that combinations improve the prediction of disease course and patient survival (2,13-15).

To examine the nature and discriminative power of genetic instability, our investigators explored the use of the UroVysion FISH test, which detects the aneusomy of chromosomes 3, 7

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and 17, and loss of the 9p21 locus, to define distinct chromosomal patterns in urine cytology samples of patients with superficial bladder cancer (16). Our pilot results suggested that the measurement of generalized chromosome polysomy may provide a genetic marker for assessing the risk of recurrence and progression in patients with superficial urothelial neoplasias.

The present study aimed to expand on the previous investigation of measuring chromosome polysomy in urine cytology by FISH to histological samples of bladder tumours and evaluate whether a combined molecular grade, composed of FISH pattern, morphometric estimates of immunohistochemical Ki-67 and p53 expression and DNA ploidy can provide tools for a more objective stratification of bladder cancer patients into low- and high-risk groups than subjective histopathological grading.

## Materials and methods

**Patients and tissues.** Routinely formalin-fixed and paraffin-embedded urinary bladder biopsy specimens from 45 consecutive patients (38 men and 7 women; mean age 68.4 years, range 45-86) with new or recurrent urothelial neoplasms diagnosed between January 1, 2004 and June 30, 2007 were investigated. Twenty-two patients were under surveillance for recurrence of superficial bladder cancer after complete transurethral resection (TUR). Cystectomy was performed in nine patients who had multiple recurrences at repeated cystoscopies. Nephrectomy was performed in two patients who developed ureteral carcinomas in addition to their bladder tumours. Tissue biopsies were obtained in different outpatient urological practices and hospitals from the federal state of North Rhine-Westphalia, West Germany and sent to the Institute of Pathology in Bonn for routine histological diagnosis.

**Follow-up.** After surgery, follow-up was performed prospectively with urine cytology and cystoscopy including directed biopsy if indicated. Twenty-two patients were eligible for disease outcome assessment, with a mean follow-up of 34.41 months (range 3-152). Cases with recurrence within 3 months of the primary diagnosis were considered to have residual primary tumours rather than true recurrences and were classified together with the diagnostic cases. To assess clinical outcome, the categories used were: i) uneventful, no signs of disease recurrence or progression during follow-up; ii) recurrence only, reappearance of neoplasia after therapy in the same stage and grade as the primary tumour; and iii) progression, disease reappearance in advanced stage and/or grade.

**Histological analysis.** H&E-stained slides were reviewed and classified independently by two pathologists (D.B. and M.B.). Staging and grading were performed according to the 2002 TNM classification (17) and the 1999 WHO system (18), respectively. In case of disagreement, slides were re-examined and re-classified until a consensus was achieved. Histological diagnoses of primary bladder tumours included papillary urothelial carcinoma of grades 1 (n=3), 2 (n=10) and 3 (n=17), while 12 cases not were graded unequivocally by histology (2 with grade 1-2 and 10 with grade 2-3). Regarding stage

distribution, pTis was found in 1, pTa in 18, pT1 in 19 and pT2 in 5 cases. Papillary urothelial neoplasm of low malignant potential (PUNLMP) was diagnosed in two patients. The characteristic histology of papillary UCs on H&E slides is shown in Fig. 1A-C.

**p53 and MIB1 immunohistochemistry and scoring.** The cases were stained for p53 (monoclonal mouse anti-human antibody clone DO 7, Ventana, Germany; ready-to-use) and Ki-67 (monoclonal mouse anti-human antibody, clone K-2, Ventana, Germany; ready-to-use) on 4  $\mu$ m serial deparaffinised and rehydrated paraffin sections following heat-induced epitope retrieval in CC1 buffer in the fully automated Ventana BenchMark XT System (Ventana Medical Systems Inc., Tucson, AZ, USA) based on the avidin-biotin peroxidase method according to standard protocols. Development was performed using the iView polymer DAB detection kit. Established positive control sections were included in each run together with tumour tissue sections mounted on the same slides.

Staining results were quantitatively analysed using bright field microscopy imaging coupled with advanced colour detection software [Automated Cellular Imaging System (ACIS), ChromaVision Medical Systems Inc., San Juan Capistrano, CA]. Signal classification was based on predetermined colour morphology and reported as a percentage of stained tumour cells, staining intensity and histoscore (percentage of tumour cells plus staining intensity). A minimum of four distinct areas from each specimen were analysed in triplicate. Data were expressed as the mean  $\pm$  SD for the analyses. Thresholds for high labelling indices were set for Ki-67 at  $\geq 10\%$  positive tumour nuclei and for p53 at 10 and 20%, according to data from the literature (10,14). Characteristic Ki-67/MIB1 and p53 immunohistochemical reactions and their morphometric evaluation results by Chroma Vision are shown in Fig. 1D-I.

**Isolation of nuclei from paraffin-embedded tumour tissues for FISH and DNA image cytometry.** Sections (50  $\mu$ m) were cut from the paraffin tissue blocks for nuclear extraction, deparaffinized in xylene (60°C overnight) and rehydrated in a series of decreasing ethanol concentrations to water. Samples were digested in pre-warmed 0.5% pepsin solution (pH 1.5) for 60 min at 37°C, washed in PBS two times by vortexing vigorously (3500 U/min for 10 min) and resuspended in 0.5 ml PBS. Two cytopspins were prepared from each sample (Hettich cytopspin; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), one for FISH and one for DNA image cytometry, and air-dried for storage.

**UroVysion FISH on paraffin-extracted nuclei from urothelial neoplasias.** The UroVysion FISH test was applied to cytopspin preparations from the paraffin-extracted nuclei of urothelial neoplasias. Laboratory procedure was performed according to the manufacturer's instructions. Briefly, specimens were fixed in methanol/acetic acid for 10 min at -20°C and digested by using 0.2 mg/ml pepsin in 0.02 N HCl for 10 min at 37°C in a humidified chamber. Subsequently, slides were washed twice in 1X phosphate-buffered saline (PBS) for 5 min each at room temperature, fixed in 4% neutral-buffered formalin/PBS for

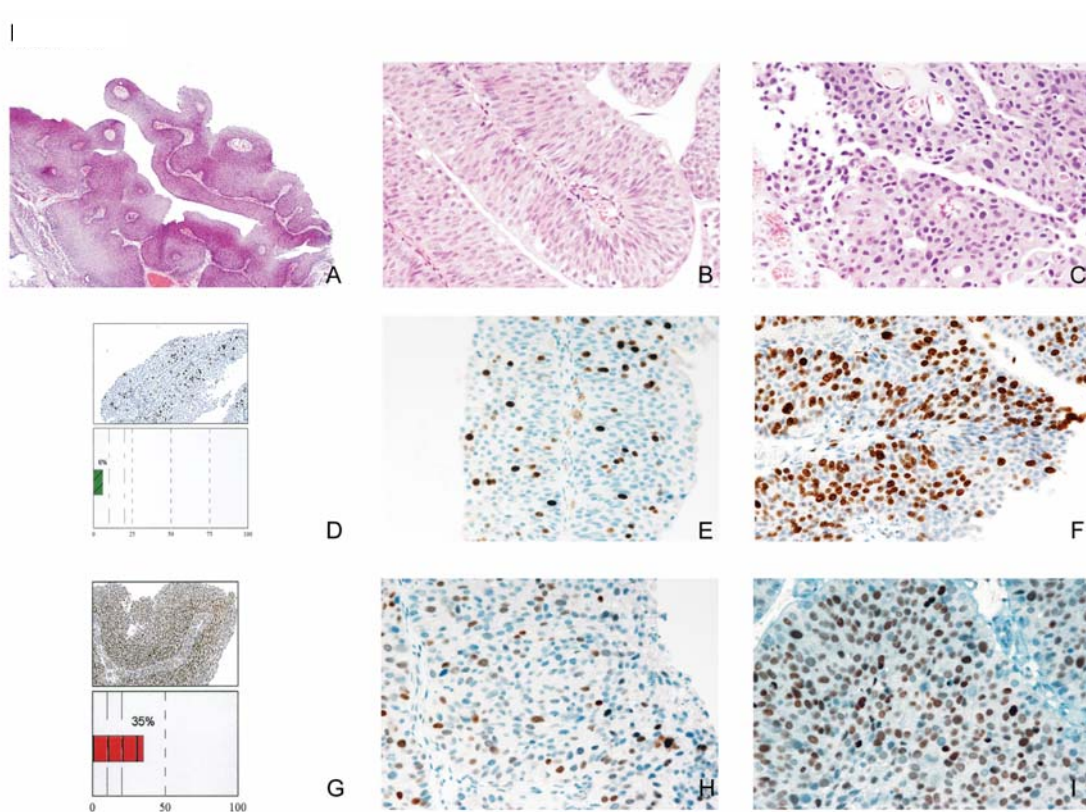


Figure 1. (A) Characteristic histology of a papillary urothelial neoplasia (magnification, x25). (B) Histology of a grade 1 papillary urothelial carcinoma (magnification, x400). (C) Histology of a grade 3 papillary urothelial carcinoma (magnification, x400). (D) Low Ki-67 LI as demonstrated by a characteristic ChromaVision panel. (E) Concurrent immunohistochemical appearance of low Ki-67 LI in a histological section of a papillary UC (magnification, x250). (F) Characteristic immunohistochemical appearance of high Ki-67 LI in a histological section of a papillary UC (magnification, x250). (G) High p53 LI as demonstrated by a characteristic ChromaVision panel. (H) Characteristic immunohistochemical appearance of low p53 LI in a histological section of a papillary UC (magnification, x250). (I) Concurrent immunohistochemical appearance of high p53 LI in a histological section of a papillary UC (magnification, x250).

5 min, washed in 1X PBS for 5 min and dehydrated in an increasing ethanol series.

Then, 10  $\mu$ l of the hybridisation mix [8  $\mu$ l hybridisation buffer LSI-WCP (Vysis), 1  $\mu$ l distilled water and 1  $\mu$ l UroVysion probe mix, consisting of directly labelled fluorescent probes to the peri-centromeric regions of chromosomes 3 (CEP-3-red), 7 (CEP-7-green) and 17 (CEP 17-aqua) and to the locus 9p21 (LSI 9p21-gold)], was placed on the target, coverslipped and sealed with rubber cement.

Following co-denaturation at 73°C for 10 min, slides were incubated at 37°C overnight in a humidified chamber. Post-hybridisation washings were performed in 2X SSC at 73°C and in 2X SSC at room temperature for 2 min each. 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was used for counterstaining.

**Evaluation of FISH signals.** Each case was evaluated by an independent scientist (H.H.), experienced with FISH analysis, who was blinded to clinical or histological findings. Slides were scored for hybridisation signals using a Zeiss Axioplan fluorescence microscope (Zeiss, Jena, Germany) with an appropriate filter set at a magnification of x400. Spot counting was performed by automatic interphase FISH analysis using the Metafer-Metacyte slide scanning system (Metasystems GmbH, Altlussheim, Germany). A minimum of 30 abnormal nuclei per slide was evaluated in each case and 30 normal-appearing urothelial or lymphoid cell nuclei in the same

cytospin were analysed for internal control. FISH results were considered positive if: i) 10 or more nuclei exhibited deletion of 9p21 (monozygous or heterozygous), ii) 10 or more nuclei exhibited polysomy of one chromosome, or iii) a minimum of 5 nuclei exhibited polysomy of >1 chromosome per cell (Vysis-Abbott Diagnostics). Tetraploid chromosomal sets were not considered as a sign of malignancy.

The mean copy number of each chromosome (CI, chromosome index; the total number of hybridisation signals divided by the total number of nuclei analysed) and the frequency of polysomies (PI, polysomy index; the percentage of scored nuclei exhibiting  $\geq 3$  chromosome copies) were calculated to estimate the degree of chromosome instability in each case.

None of the urothelial neoplasias with the disomic chromosomal pattern had negative FISH results. Therefore, FISH results were classified into 2 groups for prognostic analysis: i) low-risk, showing loss of 9p21 either as the only chromosomal aberration or accompanied by single cells (<10%) with tri- or tetrasomy/ies of either or all the chromosomes 3, 7 and 17, and ii) high-risk, revealing loss of 9p21 and >10% aneusomy/ies for either or all marker chromosomes. A characteristic panel of a Metafer-Metacyte gallery with images of selected atypical nuclei of UCs after UroVysion FISH is shown in Fig. 2A-C.

**DNA image cytometry on paraffin-extracted nuclei from urothelial neoplasias.** A second cytospin preparation of the same nuclear suspension prepared for FISH analysis was



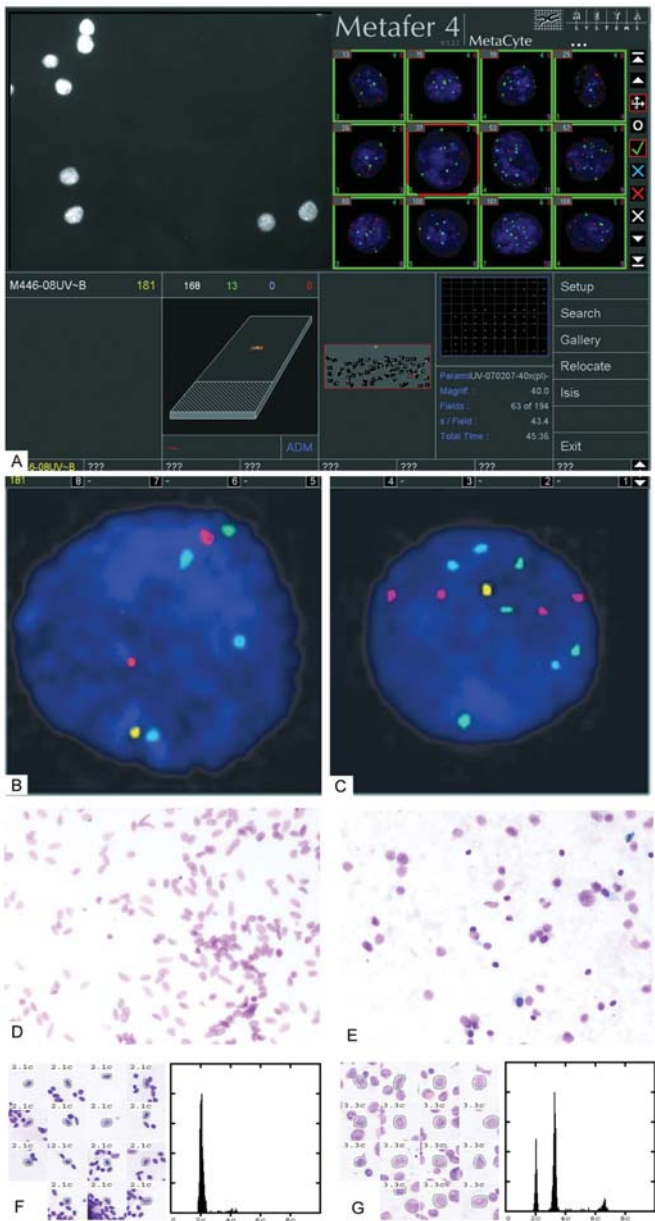


Figure 2. (A) Characteristic panel of a Metafer-Metacyte gallery with images of selected atypical nuclei after multicolour, multiprobe FISH (UroVysion). (B) UroVysion FISH result showing low-risk FISH pattern with heterozygous deletion of 9p21 (one gold signal) and disomy of chromosomes 3 (red), 7 (green) and 17 (aquamarine). (C) UroVysion FISH result showing high-risk FISH pattern with heterozygous deletion of 9p21 (one gold signal) and different polysomies of chromosomes 3 (four red signals), 7 (four green signals) and 17 (three aquamarine signals). Feulgen-stained extracted nuclei from (D) diploid and (E) aneuploid lesions (magnification, x100, respectively). Concurrent set of measured nuclei and DNA ploidy diagram from (F) diploid and (G) aneuploid lesions.

stained according to the Feulgen method. For the measurements, a PC-based image analyser (ACAS; Ahrens, Bargteheide, Germany) with special DNA software was used. To assess the diploid (2 c) range, the integrated optical density of the nuclei of 30 lymphocytes or normal urothelial cells in the cytopins was analysed. The CV value of these reference cells was not to exceed 5%. Subsequently, at least 250 tumour cell nuclei were measured selectively in each sample. Only intact, non-overlapping nuclei were included in the analysis.

Table I. Patient and tumour characteristics (n=45).

	n	%
Gender		
Male	38	84.4
Female	7	15.6
Entity		
PUNLMP	2	4.4
pTis UC	1	2.2
pTa UC	18	40.0
pT1 UC	19	42.2
pT2 UC	5	11.1
Histological grade (WHO 1999)		
1	3	7.1
1-2	2	4.8
2	10	23.8
2-3	10	23.8
3	17	40.5
FISH pattern		
Low-risk	20	44.4
High-risk	25	55.6
DNA ploidy		
Diploid	21	46.7
Non-diploid	24	53.3
p53 expression		
Negative (<10%)	16	35.6
Positive (>10%)	29	64.4
MIB1 labelling index		
Low (<10%)	14	31.1
High (>10%)	31	68.9
Molecular grade		
Low	7	15.5
High	38	74.5

PUNLMP, papillary urothelial neoplasm of low malignant potential.

Data were interpreted using the analysis software of the system. DNA histograms were classified as: i) diploid (stem line DNA peak between 1.80 and 2.20 c), ii) polyploid (at least two stem lines between 1.8-2.2 and 3.6-4.4 c, or ~8 and 16 c) and iii) aneuploid (abnormal stem line beyond diploid or polyploid peaks, and/or >10% of the nuclei showing DNA values >5 c and/or presence of single cells with DNA content >9 c). Characteristic examples of the Feulgen-stained nuclei from UCs with concurrent DNA-ploidy diagrams are shown in Fig. 2D-G.

**Molecular grading.** Based on the results of molecular tests, tumours were subdivided into: i) high molecular grade, showing aberrant expression of at least one of the four



# Relationship between tumour characteristics and molecular markers in 45 primary urothelial neoplasias.

	FISH pattern <sup>a</sup>		DNA ploidy <sup>b</sup>		p53 expression <sup>c</sup>		MIB1 LI <sup>d</sup>		Molecular grade <sup>e</sup>	
	Low-risk	High-risk	Diploid	Non-diploid	Negative <10%	Positive >10%	Negative <10%	Positive >10%	Low	High
Histology of bladder neoplasias and stage, if appropriate										
PUNLMP (n=2)	2	0	2	0	2	0	2	0	2	0
pTis (n=1)	0	1	0	1	0	1	0	1	0	1
pTa (n=18)	14	4	16	2	8	10	9	9	3	15
pT1 (n=19)	4	15	3	16	4	15	2	17	1	18
pT2 (n=5)	0	5	0	5	2	3	1	4	1	4
Total (n=45)	20	25	21	24	16	29	14	31	7	38
Grade (WHO 1999)										
1 (n=3)	2	1	3	0	2	1	3	0	1	2
1-2 (n=2)	2	0	2	0	1	1	0	2	0	2
2 (n=10)	7	3	8	2	5	5	6	4	2	8
2-3 (n=10)	6	4	5	5	2	8	2	8	1	9
3 (n=17)	1	16	1	16	4	13	1	16	1	16
Total (n=42)	18	24	19	23	14	28	12	30	5	37

<sup>a</sup>Significant direct association between chromosomal instability by FISH and tumour stage and grade (Pearson's Chi-square statistics,  $P=0.001$  and  $P=0.002$ , respectively). <sup>b</sup>Significant direct association between DNA ploidy and tumour stage and grade (Pearson's Chi-square statistics,  $P=0.000$  and  $P=0.002$ , respectively). <sup>c</sup>Lack of statistically significant association between immunohistochemical p53 expression and tumour stage and grade. <sup>d</sup>Significant direct association between MIB1 LI and tumour stage and grade (Pearson's Chi-square statistics,  $P=0.018$  and  $P=0.001$ , respectively). <sup>e</sup>Significant direct association between molecular grade and tumour stage (Pearson's Chi-square statistics,  $P=0.013$ ), no significant association with histopathological grade.

biomarkers investigated (high-risk FISH, or non-diploid DNA content, or p53 LI  $\geq 10\%$  or MIB LI  $\geq 10\%$ ) and ii) low molecular grade, with molecular patterns below the threshold for high-grade (low-risk FISH and diploid DNA content and p53 LI  $\leq 9$  and MIB LI  $\leq 9$ ).

**Statistical analysis.** Descriptive statistics for continuous measures are given as the mean and/or median with minimum and maximum values. Pearson's Chi-square test was used to compare molecular patterns with clinical and pathological findings. Correlation between variables was computed using Spearman's rank analysis. Differences in the clinical outcome between patient groups with different histopathological and molecular patterns were evaluated by the Mann-Whitney U-test.

## Results

**Patient and tumour characteristics.** The distribution of patient and tumour characteristics are shown in Table I. Primary urothelial neoplasias from 38 men and 7 women were included in the study, including 2 PUNLMPs, 19 non-invasive (intra-epithelial) (1 pTis and 18 pTa), 19 superficially invasive (pT1) and 5 deeply invasive (pT2) carcinomas. The histomorphological tumour grade was defined unequivocally in 30 neoplasias (3 grade 1, 10 grade 2 and 17 grade 3) using the 3-tired WHO 1999 classification. Ambiguous grades (2 grade

1-2 and 10 grade 2-3) were given to 12 tumours based on histomorphology alone. In contrast, molecular patterns as single variables and combined molecular grades were classified according to a 2-tired system using the 2004 WHO histopathological classification (19).

**Association of molecular patterns with pathological tumour characteristics.** The differential expression of the four biomarkers and the combined molecular grade according to the morphological stage and grade of the tumours is shown in Table II. For the four biomarkers, the prevalence of alteration gradually increased from lower stage and grade tumours to more advanced ones. Statistically significant direct association was computed between the tumour stage and chromosomal instability [FISH pattern ( $P=0.001$ )], DNA ploidy ( $P=0.000$ ), MIB1 LI ( $P=0.018$ ) and molecular grade ( $P=0.013$ ). The histopathological tumour grade was significantly associated with the FISH pattern ( $P=0.002$ ), DNA ploidy ( $P=0.002$ ) and MIB1 LI ( $P=0.001$ ). No significant association was detected between p53 expression and either morphological variables, at neither the 10 nor 20% threshold. No significant association between the histomorphological and molecular tumour grades was found.

A statistically significant correlation between the morphological and molecular markers was found for histopathological grade and MIB1 LI ( $r_s 0.475$ ,  $P<0.001$ , Spearman's rank correlation), and for tumour stage with grade, FISH pattern, DNA

Table III. Relationship of tumour characteristics and clinical outcome in 22 patients with urothelial neoplasias and follow-up.

Variable	No.	Uneventful No.	Recurrence only No.	Progression No.	Chi-square	P-value
Whole series	22	5	11	6		
Gender						
Male	20	5	10	5		
Female	2	0	1	1	0.630	NS
Stage						
PUNLMP	1	0	1	0		
pTa	12	2	7	3		
pT1	8	3	2	3		
pT2	1	0	1	0	0.615	NS
Grade (WHO 1999)						
1	1	0	1	0		
1-2	1	0	1	0		
2	7	2	3	2		
2-3	5	2	2	1		
3	7	1	3	3	0.870	NS
FISH pattern						
Low-risk	11	3	6	2		
High-risk	11	2	5	4	0.620	NS
DNA ploidy						
Diploid	12	3	7	2		
Non-diploid	10	2	4	4	0.470	NS
p53 expression						
≤10%	8	3	4	1		
>10%	14	2	7	5	0.330	NS
MIB1 LI						
≤10%	9	4	5	0		
>10%	13	1	6	6	7.410	0.025
Molecular grade						
Low	4	3	1	0		
High	18	2	10	6	2.830	0.020

NS, not significant.

ploidy and MIB1 LI (rs 0.308,  $P=0.047$ ; rs 0.551,  $P<0.000$ ; rs 0.692,  $P<0.000$  and rs 0.405,  $P<0.006$ , respectively).

*Association of tumour morphological and molecular characteristics with clinical outcome.* During clinical follow-up (mean 34.41 months and range 3-152), five patients had an uneventful outcome, 11 developed recurrences in the same stage and grade as the primary tumour and 6 showed disease progression with reappearance in advanced stage or grade. The relationship of tumour characteristics and clinical outcome is shown in Table III. A statistically significant association of outcome was found with MIB1 LI ( $P=0.025$ ) and molecular grade ( $P=0.02$ ).

Progressive tumours were characterized by an increased MIB1 LI and high molecular grade. Recurrence rates were also significantly different between groups with low and high molecular grade tumours as well as with low and high MIB1 LIs ( $P=0.007$  and  $P=0.008$ ; Mann-Whitney, respectively).

*Patient and tumour characteristics in cases with ambiguous histopathological tumour grade.* Distribution of the clinico-pathological, molecular and outcome variables in cases with ambiguous histopathological tumour grades is shown in Table IV. Six of 12 patients had clinical follow-up results. Two patients were uneventful at three months, three developed



SPANDIDOS PUBLICATIONS. Patient, tumour and clinical characteristics of 12 urothelial carcinomas with ambiguous histopathological grade to the WHO 1999 classification.

Case no.	Grade (WHO 1999)	Gender	Age	Outcome	pT stage	FISH pattern	DNA ploidy	p53 LI (%)	MIB1 LI (%)	Molecular grade
1	2-3	Male	52	Uneventful (at 3 months)	pT1	Low-risk	Diploid	5	6	Low
2	2-3	Male	66	Uneventful (at 3 months)	pT1	High-risk	Tetraploid	37	5	High
3	2-3	Male	71	Progression	pT1	Low-risk	Tetraploid	37	86	High
4	1-2	Male	45	Recurrence	pTa	Low-risk	Diploid	6	12	High
5	2-3	Male	74	Recurrence	pTa	Low-risk	Diploid	15	18	High
6	2-3	Male	72	Recurrence	pT1	High-risk	Aneuploid	74	16	High
7	2-3	Male	70	-	pT1	High-risk	Aneuploid	21	34	High
8	2-3	Male	63	-	pTa	High-risk	Diploid	15	42	High
9	2-3	Male	64	-	pTa	Low-risk	Diploid	6	32	High
10	1-2	Female	53	-	pT1	Low-risk	Diploid	84	69	High
11	2-3	Male	56	-	pTa	Low-risk	Diploid	41	30	High
12	2-3	Female	76	-	pT1	High-risk	Aneuploid	86	34	High

recurrences and one experienced tumour progression. Low molecular grade was given only to one pT1 carcinoma which had an uneventful clinical outcome. The other cases were classified as tumours with high molecular grade implicating increased risk for recurrence and progression. Although one of these cases showed an uneventful outcome, this result was yielded as early as 3 months after the operation and no further follow-up data were available. This case together with the other six diagnostic cases, all assigned high molecular grade, should be considered as anticipatory high-risk cases for tumour recurrence and progression.

## Discussion

Only a few studies have analysed the performance and prognostic value of multiple molecular markers in bladder urothelial neoplasias since the introduction of the WHO/ISUP grading scheme (now recognized as the 2004 WHO classification) (15,20). Recent studies have focused mainly on p53 and Ki-67/MIB1 expression indicating that i) the prevalence of p53 alterations increases with stage and grade (21) without being independent prognostic factors, (8) and ii) MIB1 labelling index is significantly associated with recurrence and stage progression and is an independent predictor of disease-free and overall survival, at least in pTa/pT1 bladder UCs (8,10).

This study shows that quantitative assessment of molecular biomarkers can be used to predict the clinical outcome of UCs more accurately than traditional clinicopathological parameters. In particular, the use of a 2-tiered molecular classification proved to be superior to the classic 3- or 5-tiered histological grading systems and it would be beneficial to render tumours with ambiguous pathological grade to either of the molecular grades. Though the histopathological grade was significantly related to tumour stage (rs 0.31,  $P=0.047$ ), it was not associated with clinical outcome and no significant correlation between

histological and molecular grade was found. In contrast, molecular grade showed a significant correlation with outcome (rs 0.53,  $P=0.012$ ) and tumours with stage or grade progression had high molecular grades.

Regarding single parameters, the p53 labelling index showed no diagnostic and prognostic utility in our present panel of bladder carcinomas. P53 nuclear overexpression was observed in 29 (64.4%) or 22 (48.8%) of our 45 cases depending on the cut-off levels used for a positivity of 10 or 20%, respectively. This is in agreement with published results, which encompass a range between 22-64% (21). However, we found no significant association of p53 LI in either stage and grade or clinical outcome. The proportion of tumours over-expressing nuclear p53 showed a significantly positive correlation only with the other molecular markers investigated: MIB1 LI, DNA ploidy and FISH pattern (rs 0.40,  $P=0.006$ ; rs 0.41,  $P=0.005$  and rs 0.40,  $P=0.007$ , respectively), supporting the well-established fact that p53 is a significant member in the molecular progression of bladder UC (8). Our negative results may be due to the relatively small sample size and the limited follow-up, but there may be other important issues as well, regarding counts and rates in immunohistochemistry in general. These include i) the method of counting itself, which is poorly reproducible if performed semiquantitatively (22) and ii) the selection of threshold for positivity, which varies in the literature for UC (8,10,23). In an attempt to overcome these strictly methodical problems, we determined the immunohistochemical scores for p53 and MIB1 staining by using automated cellular image analysis with a large body of well-validated evidence for objectivity and reproducibility (22,24) and performed statistical calculations with the two most frequently used cut-offs for a positivity of 10 and 20%, respectively.

The strong prognostic and predictive value of cell proliferative activity as assessed by Ki-67/MIB1 LI for the recurrence and progression of UCs has been confirmed by many



investigators (10,20,23,24). However, sampling and thresholds vary, making the comparison of results difficult. Our results are in line with previous findings of others supporting the significance of the Ki-67/MIB1 positive growth fraction as a determinant of the biological aggressiveness of UC and as a prognostic factor. Ki-67/MIB1 LI measured by automated cellular image analysis at a threshold of 10% for positivity was the only biomarker in our study which showed significant correlations with the clinicopathological tumour characteristics (stage and grade), clinical outcome (recurrence and progression) and the other molecular markers investigated (p53 LI, DNA ploidy and FISH pattern). This indicates that Ki-67/MIB1 LI may be used to stratify patients with bladder UCs into risk groups assisting therapy decisions in everyday clinical practice. This further underlines the importance of an objective and reproducible assessment of Ki-67/MIB1 LI, which can be achieved by using quantitative image morphology (22).

Deranged proliferation is a hallmark of malignancy. Cell division errors frequently lead to infidelity of mitotic chromosome segregation and result in numerical and structural chromosomal abnormalities that is probably the major cause of aneuploidy in tumour cells (25). We previously hypothesized that high-frequency polysomies of chromosomes 3, 7 and 17, in addition to 9p21 deletion detected by the UroVysion FISH-assay in urine specimens from subjects with bladder UC, may be either a reflection of aneuploidy or genomic instability. This approach proved to be very effective for predicting tumour recurrence and progression of pTa/pT1 UCs and may be a useful marker for non-invasive surveillance of patients with a history of bladder cancer (16). Our current results indicate that FISH pattern, determined in extracted nuclei of paraffin-embedded bladder UCs, is correlated with DNA ploidy (rs 0.82,  $P < 0.000$ ) and the two markers showed a highly significant correlation with tumour stage (rs 0.55 and 0.70, respectively). This is supported by the results of others (10-12,26). Though FISH pattern and DNA ploidy as single markers were not directly associated with disease outcome in the present cohort, which is probably sampling and follow-up related, but as variables of the multiparametric molecular grade, they contributed to its prognostic value.

The cumulative results of our present pilot study provide further evidence that early genetic changes during urothelial carcinogenesis and progression do not necessarily correlate with observable changes in morphology. Thus, testing for molecular alterations and ploidy in early lesions may identify patients who are at the greatest risk for recurrence and progression and lead to more definitive therapy.

We conclude that the combination of quantitative biomarkers provides significantly better, objective and reproducible prognostic tools in bladder UCs than classic clinicopathological features and indices. Although the prognostic value of the combination of complementary markers is superior to that of single parameters, the majority of information appears to reside in Ki-67/MIB1 LI. The well-established robustness and reproducibility of quantitative pathology assessments provide a solid basis for the implementation and routine use of modern pathology methods for individualized prognostication and risk-adapted treatment of patients with bladder UCs.

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