Detection of Urothelial Bladder Cancer Cells in Voided Urine Can Be Improved by a Combination of Cytology and Standardized Microsatellite Analysis

Peter J. Wild,[1] Thomas Fuchs,[3,4] Robert Stoehr,[5] Dieter Zimmermann,[1] Simona Frigerio,[1] Barbara Padberg,[1] Inbal Steiner,[8] Ellen C. Zwarthoff,[6] Maximilian Burger,[7] Stefan Denzinger,[7] Ferdinand Hofstaedter,[8,9] Glen Kristiansen,[1] Thomas Hermanns,[2] Hans-Helge Seifert,[2] Maurizio Provenzano,[2] Tullio Sulser,[2] Volker Roth,[10] Joachim M. Buhmann,[3] Holger Moch,[1] and Arndt Hartmann[5]

[1]Institute of Surgical Pathology and [2]Department of Urology, University Hospital Zurich; [3]Department of Computer Science and [4]Competence Center for Systems Physiology and Metabolic Diseases, ETH Zurich, Zurich, Switzerland; [5]Institute of Pathology, University of Erlangen, Erlangen, Germany; [6]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands; [7]Department of Urology and [8]Institute of Pathology, University of Regensburg; [9]Central Tumour Registry Regensburg, Regensburg, Germany; and [10]Computer Science Department, University of Basel, Basel, Switzerland

Abstract

Purpose: **To evaluate molecular and immunohistochemical markers to develop a molecular grading of urothelial bladder cancer and to test these markers in voided urine samples.**
Experimental Design: **255 consecutive biopsies from primary bladder cancer patients were evaluated on a tissue microarray. The clinical parameters gender, age, adjacent carcinoma *in situ*, and multifocality were collected. UroVysion fluorescence *in situ* hybridization (FISH) was done. Expression of cytokeratin 20, MIB1, and TP53 was analyzed by immunohistochemistry. *Fibroblast growth factor receptor 3* (*FGFR3*) status was studied by SNaPshot mutation detection. Results were correlated with clinical outcome by Cox regression analysis. To assess the predictive power of different predictor subsets to detect high grade and tumor invasion, logistic regression models were learned. Additionally, voided urine samples of 119 patients were investigated. After cytologic examination, urine samples were matched with their biopsies and analyzed for loss of heterozygosity (LOH), *FGFR3* mutation, polysomy, and p16 deletion using UroVysion FISH. Receiver operator characteristic curves for various predictor subsets were plotted.**
Results: **In biopsies, high grade and solid growth pattern were independent prognostic factors for overall survival. A model consisting of UroVysion FISH and *FGFR3* status (FISH + FGFR3) predicted high grade significantly better compared with a recently proposed molecular grade (MIB1 + FGFR3). In voided urine, the combination of cytology with LOH analysis (CYTO + LOH) reached the highest diagnostic accuracy for the detection of bladder cancer cells and performed better than cytology alone (sensitivity of 88.2% and specificity of 97.1%).**
Conclusions: **The combination of cytology with LOH analysis could reduce unpleasant cystoscopies for bladder cancer patients.** (Cancer Epidemiol Biomarkers Prev 2009;18(6):1798–806)

Introduction

At the time of first diagnosis, ~70% of bladder tumors are noninvasive papillary low-grade tumors (pT_a). Despite the fact that the majority of urothelial bladder tumors are clinically benign, regular cystoscopic follow-up at intervals is done in all patients with non-muscle-invasive bladder cancer after complete transurethral resection to detect recurrence and progression.

Mutations of the tumor suppressor genes TP53 and RB1 are common and have predictive value in clinical studies of invasive bladder cancer (1-3). Although TP53 alterations have been suggested as prognostic marker in pT_a tumors (4), the prognostic value of both TP53 and RB1 is restricted to invasive tumors. In non-muscle-invasive bladder cancer, homogeneous expression of cytokeratin 20 (CK20; ref. 5), lack of *fibroblast growth factor receptor 3* (*FGFR3*) mutations (6, 7), and high nuclear Ki-67 labeling index (7) show promise in predicting recurrence. Mutations in the *FGFR3* gene are very frequent in pT_a bladder tumors (~75%; refs. 7-10). Hernandez et al. have determined the frequency and the prognostic value of *FGFR3* mutations in patients with primary non-muscle-invasive bladder cancer in a large prospective study (n = 772; ref. 11). In analogy to the data presented by van Rhijn et al. (6), their findings strongly support the notion that *FGFR3* mutations characterize a subgroup of bladder cancers with good prognosis. However, there is no prospectively evaluated set of molecular markers with sufficient predictive power to select patients for a differential therapeutic approach.

Conventional urine cytology is used as a complement to cytoscopy for the detection of new bladder carcinomas

Received 2/2/09; revised 3/19/09; accepted 3/28/09; published OnlineFirst 5/19/09.
Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).
Requests for reprints: Peter J. Wild, Institute of Surgical Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland. Phone: 41-44-6332265; Fax: 41-44-6331357. E-mail: peter.wild@usz.ch

doi:10.1158/1055-9965.EPI-09-0099

Table 1. Polysomy and relative *p16* deletion in relation to clinicopathologic, molecular, and immunohistochemical markers

Variable	Categorization	Polysomy			Relative *p16* deletion		
		≤18%	>18%	*P**	≤14%	>14%	*P**
Clinicopathologic data							
Tumor stage	pT_a	62	75	**<0.001**	90	47	**<0.001**
	pT_1	5	39	17	27		
	pT_2	4	46	20	30		
	pT_3	0	2	0	2		
	pT_4	0	3	1	2		
Histologic grade	Low	64	76	**<0.001**	93	47	**<0.001**
	High	7	89	35	61		
Adjacent carcinoma *in situ*	No	69	139	**0.004**	116	92	0.228
	Yes	2	26	12	16		
Multifocality	Solitary	12	39	0.302	16	35	**<0.001**
	Multifocal	59	126	112	73		
Growth pattern	Papillary	69	124	**<0.001**	114	79	**0.003**
	Solid	2	40	14	28		
Molecular data							
FGFR3 gene	Wild-type	12	88	**<0.001**	46	54	**0.014**
	Mutation	44	49	60	33		
Immunohistochemistry							
MIB1 immunohistochemistry	≤25%	62	100	**<0.001**	94	68	0.056
	>25%	7	57	28	36		
TP53 immunohistochemistry	≤10%	64	103	**<0.001**	101	66	**0.002**
	>10%	4	59	23	40		
CK20 immunohistochemistry	Superficial staining pattern	22	25	**0.013**	35	12	**0.002**
	Negative or >10%	49	134		90	93	

*Boldface representing P values < 0.05.

and recurrences. However, application of cystoscopy every 3 to 6 months is very unpleasant for the patient. In the past, the low sensitivity of urine cytology reported in diagnosing low-grade papillary tumors has limited its use and prevented cytology from replacing cystoscopy (12). Fluorescence *in situ* hybridization (FISH)-based detection systems are currently used in conjunction with cystoscopy for the examination of bladder washings and voided urine samples. The use of UroVysion Multicolor FISH (Vysis), a FISH assay for detection of bladder cancer, based on the use of voided urine samples, has been evaluated in multiple studies (13-15). The UroVysion FISH test was the first and only test approved by the U.S. Food and Drug Administration, which uses DNA probes to identify aneuploidy for chromosomes 3, 7, and 17 and loss of the 9p21 locus in urine specimens from subjects with urothelial bladder cancer. van Rhijn et al. (16) have systematically reviewed urine markers for bladder cancer surveillance. Urinary cytology afforded a median sensitivity of 35% (range, 13-75%), whereas the median sensitivity for FISH and microsatellite analysis was 79% (range, 70-86) and 82% (range, 75-92%). Microsatellite analysis of loss of heterozygosity (LOH) and FISH were among the most promising markers for surveillance (16). We and others have previously shown that the detection of bladder carcinoma cells can be improved by standardized microsatellite analysis (17, 18). Over 93% of patients with recurrent bladder cancer disease were identified by a combination of microsatellite (LOH) analyses and cytology of their voided urine samples.

High histologic grade as a marker for chromosomal instability is the clinically most important marker for increased risk of progression to muscle-invasive disease. However, histologic grading has a high interobserver variability with varying prognostic implications (19).

The aim of the current study was to systematically evaluate a set of molecular and immunohistochemical markers to (*a*) develop a reliable molecular grading of urothelial bladder cancer and (*b*) evaluate the usefulness of these markers to detect bladder cancer cells in voided urine.

Materials and Methods

Bladder Cancer Tissue Microarray. A tissue microarray was constructed as described previously (10) from 255 consecutive, formalin-fixed, paraffin-embedded, primary urothelial bladder cancer tissues (Institute of Pathology, University of Regensburg). Clinical data were obtained from the Central Tumour Registry Regensburg and by telephone interviews (M.B. and S.D.) in case of missing data. The tissue microarray contained two tissue cores of each tumor specimen. The Institutional Review Board of the University of Regensburg approved analysis of tissues from human subjects. H&E-stained slides of all tumors were evaluated by a single surgical pathologist (A.H.). Tumor stage and grade were assigned according to International Union Against Cancer and WHO criteria (20). Invasive bladder carcinomas were graded as either low grade (G2) or high grade (G3). Growth pattern was determined for all invasive tumors (≥pT_1). Papillary growth was defined by the presence of a papillary tumor component (≥20%) with a histologic grade identical to the invasive tumor. All other tumors were considered to have a solid growth pattern. Clinicopathologic data are summarized in Supplementary Table S1. Retrospective clinical follow-up data were available regarding the endpoints recurrence-free and overall survival. The median follow-up period was 77 months (range, 0-166 months). Thirty-eight

of 215 (15%) analyzable patients died during follow-up. The median follow-up for censored patients was 84 months. Recurrences were defined as cystoscopically visible tumors (using photodynamic diagnosis with 5-aminolevulinic acid) with histologic verification.

Urine Samples. As described previously (17), voided urine samples of 119 patients scheduled for transurethral resection were prospectively collected over a period of 20 months and matched with their corresponding biopsies. Of these, 81 biopsies proved to be neoplastic on histologic examination. Characteristics are given in Supplementary Table S2. Additional 38 urine samples were collected from patients whose biopsies turned out to be histologically normal or displayed inflamed urothelium without presence of neoplastic cells. Half of these tumor-negative samples were derived from patients without previous history of bladder cancer. All urine samples (15 mL) were directly collected at the Department of Urology, University Hospital Zurich, shortly before transurethral resection. The urine samples were centrifuged at 1,300 × *g* for 10 min and sediments were immediately processed for cytologic examination and FISH analysis. This study has been approved by the local ethics committee (StV-14/2003; July 30, 2003) and informed consent was obtained from all patients.

Cytologic Examination of Urine Sediments. Urine sediments were resuspended in PBS and one to three cytospin slides were prepared from an aliquot. The slides were fixed with Cytostat 400 solution (Simat) and stained with standard Papanicolaou. A cell density between 25 and 50 cells per visual field using a ×20 objective was regarded as sufficient for analysis. Slides were reviewed in a blinded fashion by a cytopathologist (B.P.) and classified according to the following morphologic criteria: cells with severe atypia diagnostic of neoplasia (P), moderately atypical cells suspicious of neoplasia (S), cells with reactive alterations (NR), and cells with normal morphology (N).

Immunohistochemistry. Immunohistochemical studies were done as described previously (10) One surgical pathologist (A.H.) performed a blinded evaluation of the slides. Positive TP53 immunoreactivity was defined as strong nuclear staining in >10% of the tumor cells. The percentage of MIB1-positive cells of each specimen was determined as described previously (21). High MIB1 labeling index was defined if >25% of the tumor cells were positive (7). CK20 staining was defined as normal (superficial staining pattern) or abnormal (negative or >10% stained) according to Harnden et al. (5).

DNA Isolation. Genomic DNA of paraffin-embedded tumors on the tissue microarray was isolated from 1.5 mm punch biopsies of the paraffin blocks (one tissue core per case). Tumor areas were marked by a surgical pathologist (A.H.) to ensure a tumor cell content of at least 80%. DNA isolation was done using the Magna Pure DNA isolation kit (Roche) according to the manufacturer's instructions. DNA from urine samples was extracted and purified with a DNA Blood Mini-Kit (Qiagen) following instructions of the manufacturer. For the few samples containing only little DNA, at least 2 ng DNA was applied.

***FGFR3* Mutation Analysis.** *FGFR3* mutation analysis was done using the SNaPshot method (22). All mutations were verified by a second and independent SNaPshot analysis.

Table 2. Univariate analyses of factors possibly influencing recurrence-free and overall survival

Variable	Categorization	Tumor recurrence			Overall survival		
		*n**	Events	*P*†	*n**	Events	*P*†
Pathologic data							
Tumor stage	pT_a	146	72	0.7534	146	4	**<0.0001**
	pT_1	48	18	48	3		
	pT_2	56	15	56	27		
	pT_3	2	1	2	2		
	pT_4	3	0	3	2		
Histologic grade	Low	150	49	0.176	150	5	**<0.0001**
	High	105	32	105	33		
Adjacent carcinoma *in situ*	No	222	95	0.6429	222	26	**0.0001**
	Yes	33	11	33	12		
Multifocality	Unifocal tumor	53	19	0.7129	53	14	**0.0029**
	Multifocal tumor	202	87	202	24		
Growth pattern	Papillary	207	95	0.3254	207	13	**<0.0001**
	Solid	47	10	47	24		
Immunohistochemistry							
MIB1	≤25%	168	76	0.7484	168	13	**<0.0001**
	>25%	68	23	68	24		
TP53	≤10%	179	80	0.5483	179	22	**0.0161**
	>10%	66	22	66	16		
CK20	Superficial staining pattern	49	23	0.6535	49	2	**0.0155**
	Negative or >10%	192	74	192	35		
Molecular data							
FGFR3 mutational status	Wild-type	110	38	0.1382	110	24	**0.0026**
	Mutation	98	50	98	7		
Relative p16 deletion	≤14%	128	56	0.881	128	12	**0.009**
	>14%	108	40	108	22		
Polysomy	≤18%	71	31	0.958	71	3	**0.004**
	>18%	165	65		165	31	

*Only the initial biopsy of each patient is included.
†Log-rank test (two-sided); boldface representing *P* values < 0.05.

Table 3. Multivariate analysis of factors possibly influencing overall survival (*n* = 186)

Variable	Categorization		Global *P*	Reverse selection (limit *P* = 0.1)	
				Hazard ratio (95% confidence interval)	*P*
Pathologic data					
Tumor stage	pT_a	0	0.558	—	
	pT_{1-4}	1			
Histologic grade	Low	0	0.199	6.608 (1.929-22.633)	**0.003**
	High	1			
Adjacent carcinoma *in situ*	No	0	0.516	—	
	Yes	1			
Multifocality	Unifocal tumor	0	0.304	—	
	Multifocal tumor	1			
Growth pattern	Papillary	0	**0.002***	4.804 (1.959-11.783)	**0.001**
	Solid	1			
MIB1 immunohistochemistry	≤25%	0	0.823	—	
	>25%	1			
TP53 immunohistochemistry	≤10%	0	0.106	0.488 (0.213-1.114)	0.088
	>10%	1			
FGFR3 gene	Wild-type	0	0.199	—	
	Mutation	1			
CK20 immunohistochemistry	Superficial staining pattern	0	0.585	—	
	Negative or >10%	1			
Relative *p16* deletion	≤14%	0	0.945	—	
	>14%	1			
Polysomy	≤18%	0	0.918	—	
	>18%	1			

*Boldface representing *P* values < 0.05.

FISH Analysis of Paraffin Specimens. Multicolor FISH was done using the UroVysion probe set (Abbott Laboratories) according to the manufacturer's instructions to assess aberrations of chromosomes 3, 7, and 17 by centromeric probes and to detect relative deletions of *p16* on locus 9p21 (23). For each case, 50 nuclei were selected for scoring according to morphologic criteria using 4′,6-diamidino-2-phenylindole staining. Only nonoverlapping intact nuclei were scored. Clearly distinguishable nonurothelial cells were disregarded. All hybridizations were evaluated by two investigators (R.S. and I.S.) with random quality control checks (A.H.). Each cell was simultaneously analyzed for centromeric signals of chromosomes 3, 7, and 17 and the *p16* locus on 9p21. A cell was considered aberrant if at least one of three centromeric signals was amplified (>2 signals per cell) or if 9p21 was deleted. Polyploid cells (4 signals of all the three probes) were regarded normal (euploid). A relative deletion of the *p16* locus (*CDKN2A*) was recognized if the signal number of 9p21 was >1 unit lower than the mean value of the centromeric signals. Based on the occurrence of polysomy and deletions of 9p21 in non-tumor-associated bladder urothelium of patients with benign prostatic hyperplasia (n = 10), a cutoff was defined using three times the SD (23). Accordingly, a case was considered aberrant if >9 cells of 50 showed polysomy (>18% of the cells). A sample was considered carrying a deletion of *p16* if >7 of 50 cells (>14% of the cells) showed a relative deletion of 9p21.

FISH Analysis of Voided Urine Samples. In each case, 25 selected cells were analyzed. The cell selection criteria included patchy and lighter nuclear 4′,6-diamidino-2-phenylindole staining, nuclear enlargement, irregular nuclear contour, and presence in a small cluster. Overlapping cells were not analyzed. Samples were scored as FISH positive, if ≥4 cells showed at least 3 copies of any of the centromeric signals for chromosomes 3, 7, and 17 and if ≥12 cells displayed a homozygous loss of 9p21 (17). Due to technical reasons, only 2 of the 38 (5%) nonneoplastic urine samples were analyzable with FISH.

Statistical Analysis. Statistical analyses were completed using SPSS version 16.0 (SPSS) and R (24). Differences were considered significant if $P < 0.05$. All samples were considered independent.

Associations between measured parameters were obtained by applying χ^2 and two-sided Fisher's exact tests. The Kaplan-Meier method was used to compare curves for the different variables with regard to recurrence-free and overall survival, with significance evaluated by two-sided log-rank statistics. For the analysis of recurrence-free survival, patients were censored at the date when cystectomy was done or at the time of their last tumor-free clinical follow-up appointment. For survival analysis, patients were censored at the time of their last clinical follow-up appointment. Cox proportional hazard ratios were estimated to obtain risks of death and to find independent prognostic factors in a multivariate model. Limit for reverse selection procedures was $P = 0.1$.

To assess the predictive power of different predictor subsets, a logistic regression model was learned for each set. Cross-validation was used to validate the predictive power of the models. Therefore, 70% of the samples were drawn at random to form the training set on which a model was learned, which then was tested on the 30% of bag samples. This procedure was repeated 100 times for each model to get estimates for the prediction error. Student's t test was employed to quantify differences between the error distributions of different models. Data from voided urine samples were described by plotting receiver operator characteristic (ROC) curves of the posterior probability for various predictor subsets. The best operation point was highlighted and the corresponding sensitivity, specificity, and positive and negative predictive values (PV+, PV-) were reported.

Results

Tissue Microarray Study of Urinary Bladder Cancer. *Immunohistochemical and Molecular Markers.* The prognostic effect of the UroVysion kit in concert with four previously described molecular markers (*FGFR3*, CK20, MIB1, and TP53) was investigated retrospectively. Investigation of UroVysion FISH in a series of 255 primary urothelial bladder cancers using tissue microarray technology was informative in 92.5% (236 of 255) of the cases. Cases of noninterpretable results were due to poor technical quality or lack of epithelial cell content. Polysomy of at least one chromosome was found in 69.9% (165 of 236) and a relative deletion of 9p21 in 45.8%

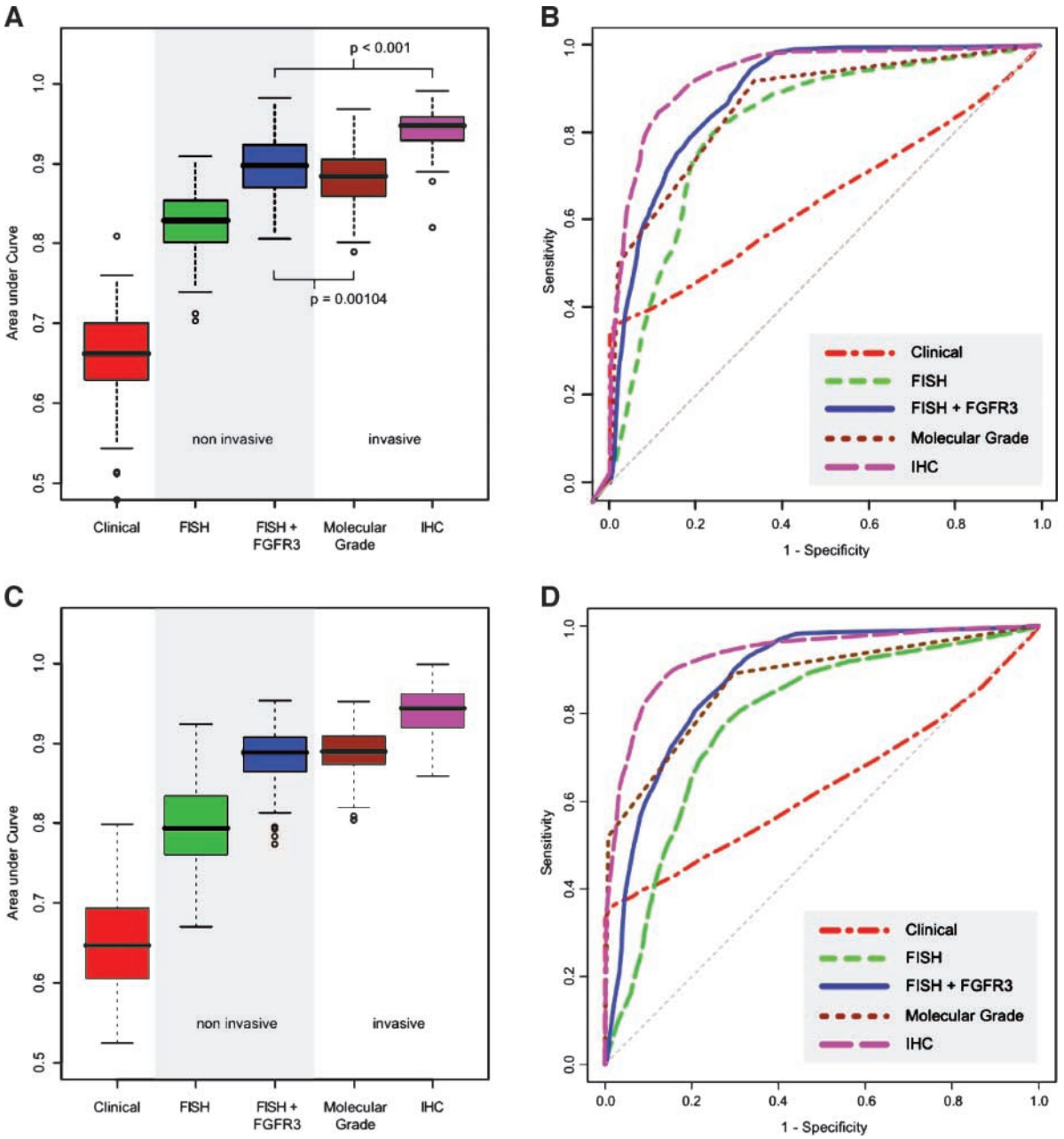

Figure 1. **A** to **D,** analysis of the prediction performance of the three models based on noninvasive predictors (CLINICAL, FISH, and FISH + FGFR3) and the two models based on invasive predictors (molecular grade and immunohistochemistry). The box plots show the area under the ROC curve to predict high histologic grade (**A** and **B**) and infiltrative growth (stage $\geq pT_1$; **C** and **D**) based on 100 cross-validation experiments. Corresponding ROC curves for the three models based on noninvasive predictors (CLINICAL, FISH, and FISH + FGFR3) and the two models based on invasive predictors (molecular grade and immunohistochemistry). The ROC curves plotted are generated by varying the threshold of the logistic regression model log(p(x)/(1-p(x))) = b_0+sum_{i = 1} ^{k}b_k*x_k and are the average curves based on 100 cross-validation experiments.

Table 4. FGFR3 analysis of voided urine samples in relation to histology, cytology, and molecular analyses

Variable	Categorization	*FGFR3* analysis		
		Wild-type	Mutation	*P**
Cystoscopically obtained biopsies				
Histologic diagnosis	Nonmalignant	33	1	**0.005**
	Malignant	51	17	
Voided urine samples				
Cytologic diagnosis	Negative cytology (N)	35	0	**<0.001**
	Atypical cytology, not specified (NR)	20	4	
	Atypical cytology, suspicious (S)	12	6	
	Positive cytology (P)	17	8	
LOH	No LOH	45	4	**0.032**
	Suspected LOH	5	1	
	LOH	34	13	
UroVysion FISH	No polysomy or loss of p16	11	2	0.290
	Polysomy or loss of p16	20	10	

*Fisher's exact test, two-sided; boldface representing P values < 0.05.

(108 of 236) of urothelial neoplasms. Results of *FGFR3* mutation analysis and MIB1, TP53, and CK20 immunohistochemistry have been published previously (10) and are given in Supplementary Tables 1 and 3.

Table 1 shows the association of UroVysion FISH results with clinicopathologic, immunohistochemical, and molecular parameters. Polysomy and relative *p16* deletion was significantly associated with high tumor stage, high grade, and solid growth pattern. Almost all cases with adjacent carcinoma *in situ* showed polysomy in at least one chromosome ($P = 0.004$). These data confirm that polysomy and relative *p16* deletions are associated with adverse histopathologic characteristics. Interestingly, a relative *p16* deletion was predominantly found in solitary compared with multifocal urothelial bladder tumors ($P < 0.001$). As expected, polysomy and relative *p16* deletion were significantly associated with wild-type *FGFR3* status, high proliferation, high TP53 immunoreactivity, and abnormal CK20 staining pattern (Table 1).

Molecular and Immunohistochemical Markers and Disease Course. The end points in the retrospective tissue microarray study were recurrence-free and overall survival. Patients with bladder tumors showing relative *p16* deletion or polysomy had a significantly shorter overall survival. Table 2 shows univariate P values for all variables investigated. None of the parameters showed prognostic effect for tumor recurrence.

We investigated the association between the molecular markers and overall survival more closely by adjusting a Cox regression model (Table 3). In the global model (including tumor stage, grade, adjacent carcinoma *in situ*, multifocality, growth pattern, *FGFR3* status, MIB1 immunohistochemistry, TP53 immunohistochemistry, CK20 staining pattern, polysomy, and relative p16 deletion), only solid growth pattern proved to be an independent predictor of shorter overall survival ($P = 0.002$). After stepwise reverse selection (limit $P = 0.1$), histologic grade, growth pattern, and TP53 immunohistochemistry remained in the model. The estimated probability of death was at least six times higher in patients with high-grade bladder cancer than that in patients with low-grade cancers ($P = 0.003$).

Assuming different model constructs, the following conclusions can be drawn: (*a*) histologic grade (high versus low grade) and growth pattern (solid versus papillary) are independent prognostic factors for overall survival and (*b*) relative p16 deletion and polysomy cannot be considered independent prognostic factors for the survival probability of bladder cancer patients.

Model Comparison. Given the prognostic effect of histologic grade, sensitivity and specificity for the detection of high-grade tumors were calculated. In this study, we compared five different models for their power to predict the surrogate markers high grade and infiltrative tumor growth (stage $\geq pT_1$) using a logistic regression model. The first model consisted of the clinical parameters sex, age, adjacent carcinoma *in situ*, and multifocality (CLINICAL). The second model comprised polysomy and relative p16 deletion (FISH). The third model extends the FISH model for the FGFR3 mutational status (FISH + FGFR3). The latter two models were constructed with markers, which could also be measured noninvasively using urine. The fourth model was the molecular grading model from van Rhijn et al. (7) and consisted of MIB1 immunohistochemistry and FGFR3 mutational status (molecular grade). The last model consisted of the immunohistochemical markers MIB1, TP53, and the CK20 pattern.

Targeting high tumor grade, the (noninvasive) FISH + FGFR3 model performs slightly better ($P = 0.001$) than the molecular grading model from van Rhijn et al. (7) with respect to the area under curve (AUC). Both have an AUC of ~0.9 as shown in Fig. 1A and B. Observing the ROC curves in Fig. 1B in detail, it can be seen that on average FISH + FGFR3 is more sensitive and that van Rhijn et al. molecular grading is more specific regarding high grade. The classic immunohistochemical markers were superior to FISH + FGFR3 ($P < 0.001$) and the molecular grade ($P < 0.001$). The clinical markers alone (CLINICAL) perform worse than all other discussed models. FISH analysis alone failed only in 4 of 96 high-grade bladder cancer cases (3 pT_1 and 1 pT_2 tumor). All other high-grade tumors were FISH positive.

Targeting infiltrative tumor growth (stage $\geq pT_1$), the FISH + FGFR3 model and the molecular grading model performed equally well with respect to the AUC (0.9; Fig. 1C and D). Again, classic immunohistochemistry markers are superior to FISH + FGFR3 and the molecular grade.

Our results show that a model consisting of UroVysion FISH and FGFR3 status (FISH + FGFR3) can predict high

grade just as well as the molecular grade proposed by van Rhijn et al. (7) and feature a higher sensitivity. Although a model based on the classic immunohistochemical markers is still the most powerful regarding the target high tumor grade, our FISH + FGFR3 model reaches nearly the same accuracy (0.9 versus 0.95 AUC) only with markers that could be measured with noninvasive techniques using urine samples instead of paraffin-embedded specimens. Parameters and *P* values of the FISH + FGFR3 logistic regression model are given in Supplementary Table S4. The model contained polysomy, relative p16 deletion, and *FGFR3* mutational status as predictors from which polysomy and *FGFR3* status are significant for the prediction of high grade.

Voided Urine Study. An independent set of 119 voided urine samples were investigated to estimate the diagnostic power of the different assays. After cytologic examination, voided urine samples were matched with corresponding biopsies and analyzed for LOH, *FGFR3* mutation, polysomy, and relative p16 deletion using UroVysion FISH. A mutated *FGFR3* gene was found significantly more frequent in patients with malignant disease ($P = 0.005$; Table 4). Only one patient with a normal biopsy was found to have a urine specimen with mutated *FGFR3*. Of note is that the same patient developed a pT_a G2 bladder tumor with mutated *FGFR3* within the same year of follow-up. Mutated *FGFR3* was significantly associated with a positive cytologic result ($P < 0.001$; Table 4). None of the cytologically negative cases displayed a *FGFR3* mutation. Separate results of the analysis of voided urine samples for patients with high-grade ($n = 40$) and low-grade ($n = 41$) bladder tumors are given in Supplementary Table S5.

Sensitivity, specificity, and positive and negative predictive values for the detection of bladder cancer cells in voided urine were calculated (Fig. 2A-F). Six different models were investigated for their power to predict bladder cancer cells in urine. The three methods (CYTO, LOH, and FGFR3) were tested individually (Fig. 2A-C). Additionally, *FGFR3* and LOH analysis were tested in combination with cytology (CYTO + FGFR3, and CYTO + LOH). Targeting neoplastic cells, the combination of cytology with LOH and *FGFR3* analysis performed equally well, respectively. Observing the ROC curves in Fig. 2D and E in detail, it can be seen that on average CYTO + FGFR3 is slightly more sensitive and CYTO + LOH is more specific for the detection of bladder cancer cells.

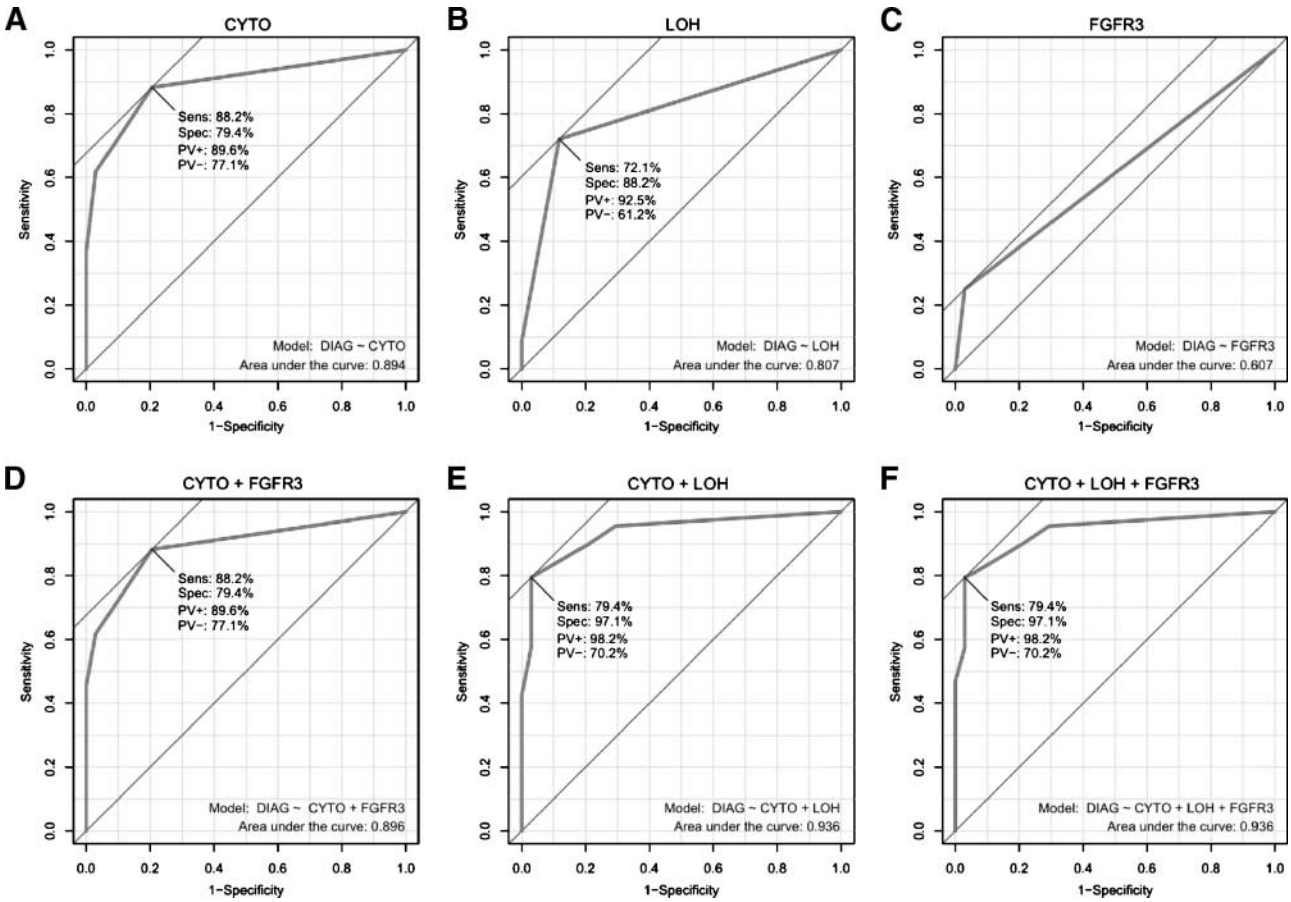

Figure 2. **A** to **F,** ROC curves for six different models to predict bladder cancer cells in a set of prospectively collected voided urine samples. The three methods (CYTO, LOH, and FGFR3) were tested individually for their ability to detect bladder cancer cells in urine (**A-C**). Additionally, *FGFR3* and LOH analysis were tested in combination with cytology (**D** and **E**). The combination of the three techniques (CYTO + LOH + FGFR3) did not add significant diagnostic advantage (**F**).

The combination of the three techniques (CYTO + LOH + FGFR3) did not add significant diagnostic advantage (Fig. 2F) when compared with the dual models. Results of the FISH assay could not be included in the logistic regression analysis for cancer cell detection because only 2 of the 38 (5%) nonneoplastic urine samples were analyzable with FISH. Sixty-nine percent of the urine samples from patients with a malignant bladder biopsy were UroVysion FISH positive. Measures of the performance of the various assays for the detection of high-grade versus non-high-grade tumors are provided in Supplementary Fig. S1A to F.

Discussion

In this study, we show that the combination of classic cytology with LOH analysis reaches the highest diagnostic accuracy for the detection of urothelial bladder cancer cells in voided urine samples.

To analyze large numbers of bladder cancer specimens, we first evaluated a tissue microarray comprising 255 consecutive, primary bladder cancers. In our survey, solid growth pattern and high histologic grade were the most important prognostic factor for overall survival. However, histologic grading has a high interobserver variability with varying prognostic implications (19). Burger et al. (25) have shown that the current WHO classification (20) reflects the outcome of bladder cancer patients more accurately than the 1973 classification system (26). The authors concluded that novel methods including molecular markers need to be evaluated for clinical use. In a second study on urothelial bladder cancer from the same group (n = 221), Burger et al. (25) prospectively investigated the prognostic value of the WHO 1973 and 2004 grading systems and biomarkers *FGFR3*, CK20, and Ki-67. They found that both grading systems contribute valuable independent information. Interestingly, combining WHO 2004 grading with *FGFR3* status allowed a better risk stratification for patients with high-grade non-muscle-invasive urothelial bladder cancer.

A set of molecular and immunohistochemical markers was evaluated to develop a reliable and objective grading systems of urothelial bladder cancer. A model consisting of UroVysion FISH and *FGFR3* status (FISH + FGFR3) predicted high grade significantly better compared with the molecular grade proposed by van Rhijn et al. (ref. 7; Fig. 1A and B).

In general, urethrocystoscopy (every 3-4 months for the first 2 years and longer intervals in subsequent years) remains the standard of care for the detection and follow-up of urothelial bladder cancer. Interestingly, van der Aa et al. (27) have assessed the discomfort and pain reported during follow-up of patients (n = 220) with non-muscle-invasive low-grade urothelial bladder cancer comparing urethrocystoscopy and surveillance by microsatellite analysis. According to van der Aa et al., periodic urethrocystoscopy caused pain and discomfort in about a third of patients, whereas the burden of microsatellite analysis appeared fully attributable to the waiting time for the test result. The authors concluded that less invasive surveillance tests are urgently needed (27).

But can the results of our aforementioned tissue microarray study be used for the detection of neoplastic cells in voided urine? To address this question, we estimated the diagnostic power for the detection of bladder cancer cells in 119 voided urine samples using LOH and *FGFR3* analysis, UroVysion FISH, and cytology as predictors. We could show that the combination of classic cytology with LOH analysis (CYTO + LOH) significantly increased the accuracy to detect malignant urothelial cells in voided urine (Fig. 2E). In our study, sensitivity and specificity of conventional cytology was already 88.2% and 79.4% (AUC = 0.894; Fig. 2A). Using a combination of cytology and *FGFR3* analysis (CYTO + FGFR3), sensitivity and specificity could not be increased (Fig. 2D). The combination of cytology with microsatellite analysis (CYTO + LOH) was able to increase specificity (97.1%) and the area under the ROC curve (Fig. 2D). However, sensitivity slightly decreased to 79.4%. Combination of the three techniques (CYTO + LOH + FGFR3) did not add significant diagnostic advantage (Fig. 2F).

These results are contrary to data published by van Rhijn et al. (28) who have also combined LOH and *FGFR3* mutation analysis (molecular grade) for the detection of urothelial cancer cells in voided urine. After cytologic examination, an independent set of voided urine samples was matched with corresponding biopsies and analyzed for LOH, *FGFR3* mutation, polysomy, and p16 deletion using UroVysion FISH. Combining results of LOH and *FGFR3* mutation analysis, the sensitivity of the combined approach increased to 89% and was superior to the sensitivity of conventional cytology for every clinical subdivision (28). In our study, however, sensitivity and specificity of conventional cytology were already very high (88.2% and 79.4%). The area under the ROC curve in Fig. 2A (AUC 0.894) could only be increased by adding the results of the microsatellite analysis (AUC 0.936; Fig. 2E).

Recently, van der Aa et al. (18) have reported the results of a longitudinal prospective multicenter trial for surveillance of patients with low-grade non-muscle-invasive urothelial cancer using microsatellite analysis (n = 228). The authors concluded that microsatellite analysis on voided urine samples is not sufficiently sensitive to recommend implementation in routine clinical practice.

Classic cytology is still regarded as an important adjunct to urethrocystoscopy. Cytologic examination of voided urine is cheap, established in almost every pathology department, and should be part of any bladder cancer surveillance protocol. However, application of urine cytology is operator-dependent and can be hampered by the low sensitivity for low-grade lesions (29). In contrast to our study, simultaneous cytologic examinations were not taken into account by van der Aa et al. when calculating sensitivity and specificity of the various tests (18, 28).

The combination of cytology with LOH analysis reached the highest diagnostic accuracy for the detection of urothelial bladder cancer cells in voided urine samples. A monitoring scheme alternating invasive cystoscopy with a combination of noninvasive techniques (including classic urine cytology and LOH analysis) could reduce unpleasant interventions and improve follow-up compliance of patients with recurrent urothelial bladder cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Monika Kerscher, Nina Nieβl, Anne Pietryga-Krieger, and Rudolf Jung for excellent technical assistance and Armin Pauer (Central Tumour Registry, Regensburg) for help in obtaining the clinical data.

References

1. Cordon-Cardo C, Zhang ZF, Dalbagni G, et al. Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors. Cancer Res 1997;57:1217–21.
2. Cote RJ, Dunn MD, Chatterjee SJ, et al. Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Res 1998;58:1090–4.
3. Masters JR, Vani UD, Grigor KM, et al. Can p53 staining be used to identify patients with aggressive superficial bladder cancer. J Pathol 2003;200:74–81.
4. Sarkis AS, Dalbagni G, Cordon-Cardo C, et al. Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. J Natl Cancer Inst 1993;85:53–9.
5. Harnden P, Mahmood N, Southgate J. Expression of cytokeratin 20 redefines urothelial papillomas of the bladder. Lancet 1999;353:974–7.
6. van Rhijn BW, Lurkin I, Radvanyi F, Kirkels WJ, van der Kwast TH, Zwarthoff EC. The fibroblast growth factor receptor 3 (FGFR3) mutation is a strong indicator of superficial bladder cancer with low recurrence rate. Cancer Res 2001;61:1265–8.
7. van Rhijn BW, Vis AN, van der Kwast TH, et al. Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912–21.
8. Billerey C, Chopin D, Aubriot-Lorton MH, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pT_a) tumors. Am J Pathol 2001;158:1955–9.
9. van Rhijn BW, Montironi R, Zwarthoff EC, Jobsis AC, van der Kwast TH. Frequent FGFR3 mutations in urothelial papilloma. J Pathol 2002; 198:245–51.
10. van Oers JM, Wild PJ, Burger M, et al. FGFR3 mutations and a normal CK20 staining pattern define low-grade noninvasive urothelial bladder tumours. Eur Urol 2007;52:760–8.
11. Hernandez S, Lopez-Knowles E, Lloreta J, et al. Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. J Clin Oncol 2006;24:3664–71.
12. Brown FM. Urine cytology. It is still the gold standard for screening. Urol Clin North Am 2000;27:25–37.
13. Placer J, Espinet B, Salido M, Sole F, Gelabert-Mas A. Clinical utility of a multiprobe FISH assay in voided urine specimens for the detection of bladder cancer and its recurrences, compared with urinary cytology. Eur Urol 2002;42:547–52.
14. Halling KC, King W, Sokolova IA, et al. A comparison of cytology and fluorescence *in situ* hybridization for the detection of urothelial carcinoma. J Urol 2000;164:1768–75.
15. Bubendorf L, Grilli B, Sauter G, Mihatsch MJ, Gasser TC, Dalquen P. Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. Am J Clin Pathol 2001;116: 79–86.
16. van Rhijn BW, van der Poel HG, van der Kwast TH. Urine markers for bladder cancer surveillance: a systematic review. Eur Urol 2005;47: 736–48.
17. Frigerio S, Padberg BC, Strebel RT, et al. Improved detection of bladder carcinoma cells in voided urine by standardized microsatellite analysis. Int J Cancer 2007;121:329–38.
18. van der Aa MN, Zwarthoff EC, Steyerberg EW, et al. Microsatellite analysis of voided-urine samples for surveillance of low-grade non-muscle-invasive urothelial carcinoma: feasibility and clinical utility in a prospective multicenter Study (Cost-Effectiveness of Follow-up of Urinary Bladder Cancer Trial [CEFUB]). Eur Urol. 2008 May 15. [Epub ahead of print].
19. Tosoni I, Wagner U, Sauter G, et al. Clinical significance of interobserver differences in the staging and grading of superficial bladder cancer. BJU Int 2000;85:48–53.
20. Eble JN, Sauter G, Epstein JI, Sesterhenn I, editors. World Health Organization classification of tumours. Pathology and genetics of tumours of the urinary system and male genital organs. Lyon: IARC Press; 2004.
21. Nocito A, Bubendorf L, Maria Tinner E, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Pathol 2001;194:349–57.
22. van Oers JM, Lurkin I, van Exsel AJ, et al. A simple and fast method for the simultaneous detection of nine fibroblast growth factor receptor 3 mutations in bladder cancer and voided urine. Clin Cancer Res 2005;11:7743–8.
23. Schwarz S, Rechenmacher M, Filbeck T, et al. Value of multicolor Fluorescence *in situ* hybridization (UroVysion-TM) in the differential diagnosis of flat urothelial lesions. J Clin Pathol 2008;61:272–7.
24. Ihaka R, Gentleman R. R: a language for data analysis and graphics. J Comput Graph Stat 1996;5:299–314.
25. Burger M, Denzinger S, Wieland WF, Stief CG, Hartmann A, Zaak D. Does the current World Health Organization classification predict the outcome better in patients with noninvasive bladder cancer of early or regular onset. BJU Int 2008;102:194–7.
26. Mostofi FK, Sorbin LH, Torloni H. Histological typing of urinary bladder tumours. International classification of tumours. Vol. 19. Geneva: WHO; 1973.
27. van der Aa MN, Steyerberg EW, Sen EF, et al. Patients' perceived burden of cystoscopic and urinary surveillance of bladder cancer: a randomized comparison. BJU Int 2008;101:1106–10.
28. van Rhijn BW, Lurkin I, Chopin DK, et al. Combined microsatellite and FGFR3 mutation analysis enables a highly sensitive detection of urothelial cell carcinoma in voided urine. Clin Cancer Res 2003;9:257–63.
29. Sherman AB, Koss LG, Adams SE. Interobserver and intraobserver differences in the diagnosis of urothelial cells. Comparison with classification by computer. Anal Quant Cytol 1984;6:112–20.

Cancer Epidemiology, Biomarkers & Prevention

Correction: Detection of Urothelial Bladder Cancer Cells in Voided Urine can be Improved by a Combination of Cytology and Standardized Microsatellite Analysis

In this article (1), which was published in the June 2009 issue of *Cancer Epidemiology, Biomarkers & Prevention*, data regarding relative p16 deletion in Table 1 and regarding overall survival in Table 2 were partly displaced. The correct tables are shown on the next page.

Table 1. Polysomy and relative *p16* deletion in relation to clinico-pathologic, molecular and immunhistochemical markers

Variable	Categorisation	Polysomy			Relative *p16* deletion		
		≤18%	>18%	*P**	≤14%	>14%	*P**
Clinico-pathologic data:							
Tumour stage							
	pTa	62	75	**<0.001**	90	47	**<0.001**
	pT1	5	39		17	27	
	pT2	4	46		20	30	
	pT3	0	2		0	2	
	pT4	0	3		1	2	
Histologic grade							
	low grade	64	76	**<0.001**	93	47	**<0.001**
	high grade	7	89		35	61	
Adjacent carcinoma *in situ*							
	no	69	139	**0.004**	116	92	0.228
	yes	2	26		12	16	
Multifocality							
	solitary	12	39	0.302	16	35	**<0.001**
	multifocal	59	126		112	73	
Growth pattern							
	papillary	69	124	**<0.001**	114	79	**0.003**
	solid	2	40		14	28	
Molecular data:							
FGFR3 gene							
	wild-type	12	88	**<0.001**	46	54	**0.014**
	mutation	44	49		60	33	
Immunohistochemistry:							
MIB1 IHC							
	≤25%	62	100	**<0.001**	94	68	0.056
	>25%	7	57		28	36	
TP53 IHC							
	≤10%	64	103	**<0.001**	101	66	**0.002**
	>10%	4	59		23	40	
CK20 IHC							
	superficial staining pattern	22	25	**0.013**	35	12	**0.002**
	negative or >10%	49	134		90	93	

*Bold face representing *P*-values <0.05.

Table 2. Univariate analyses of factors possibly influencing recurrence-free and overall survival

Variable	Categorisation	Tumor recurrence			Overall survival		
		*n**	events	*P*†	*n**	events	*P*†
Pathologic data:							
Tumour stage							
	pTa	146	72	0.7534	146	4	**<0.0001**
	pT1	48	18		48	3	
	pT2	56	15		56	27	
	pT3	2	1		2	2	
	pT4	3	0		3	2	
Histologic grade							
	low grade	150	49	0.176	150	5	**<0.0001**
	high grade	105	32		105	33	
Adjacent carcinoma *in situ*							
	no	222	95	0.6429	222	26	**0.0001**
	yes	33	11		33	12	
Multifocality							
	unifocal tumor	53	19	0.7129	53	14	**0.0029**
	multifocal tumor	202	87		202	24	
Growth pattern							
	papillary	207	95	0.3254	207	13	**<0.0001**
	solid	47	10		47	24	
Immunohistochemistry:							
MIB1							
	≤25%	168	76	0.7484	168	13	**<0.0001**
	>25%	68	23		68	24	
TP53							
	≤10%	179	80	0.5483	179	22	**0.0161**
	>10%	66	22		66	16	
CK20							
	superficial staining pattern	49	23	0.6535	49	2	**0.0155**
	negative or >10%	192	74		192	35	
Molecular data:							
FGFR3 mutational status							
	wild type	110	38	0.1382	110	24	**0.0026**
	mutation	98	50		98	7	
Relative p16 deletion							
	≤14%	128	56	0.881	128	12	**0.009**
	>14%	108	40		108	22	
Polysomy							
	≤18%	71	31	0.958	71	3	**0.004**
	>18%	165	65		165	31	

*Only the initial biopsy of each patient is included.
†Log Rank test (2-sided); bold face representing *P*-values <0.05.

Reference

1. Wild PJ, Fuchs T, Stoehr R, Zimmermann D, Frigerio S, Padberg B, Steiner I, Zwarthoff EC, Burger M, Denzinger S, Hofstaedter F, Kristiansen G, Hermanns T, Seifert HH, Provenzano M, Sulser T, Roth V, Buhmann JM, Moch H, Hartmann A. Detection of urothelial bladder cancer cells in voided urine can be improved by a combination of cytology and standardized microsatellite analysis. Cancer Epidemiol Biomarkers Prev 2009;18:1798–806.

doi: 10.1158/1055-9965.EPI-10-0005

Detection of Urothelial Bladder Cancer Cells in Voided Urine Can Be Improved by a Combination of Cytology and Standardized Microsatellite Analysis

Peter J. Wild, Thomas Fuchs, Robert Stoehr, et al.

Cancer Epidemiol Biomarkers Prev 2009;18:1798-1806.