Combined Microsatellite and *FGFR3* Mutation Analysis Enables a Highly Sensitive Detection of Urothelial Cell Carcinoma in Voided Urine[1]

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ABSTRACT

Purpose: ***Fibroblast growth factor receptor 3 (FGFR3)*** **mutations were reported recently at a high frequency in low-grade urothelial cell carcinoma (UCC). We investigated the feasibility of combining microsatellite analysis (MA) and the *FGFR3* status for the detection of UCC in voided urine.**

Experimental Design: **In a prospective setting, 59 UCC tissues and matched urine samples were obtained, and subjected to MA (23 markers) and *FGFR3* mutation analysis (exons 7, 10, and 15). In each case, a clinical record with tumor and urine features was provided. Fifteen patients with a negative cystoscopy during follow-up served as controls.**

Results: **A mutation in the *FGFR3* gene was found in 26 (44%) UCCs of which 22 concerned solitary pTaG1/2 lesions. These mutations were absent in the 15 G3 tumors. For the 6 cases with leukocyturia, 46 microsatellite alterations were found in the tumor. Only 1 of these was also detected in the urine. This was 125 of 357 for the 53 cases without leukocyte contamination. The sensitivity of MA on voided urine was lower for *FGFR3*-positive UCC (15 of 21; 71%) as compared with *FGFR3* wild-type UCC (29 of 32; 91%). By including the *FGFR3* mutation, the sensitivity of molecular cytology increased to 89% and was superior to the sensitivity of morphological cytology (25%) for every clinical subdivision. The specificity was 14 of 15 (93%) for the two (molecular and morphological) cytological approaches.**

Conclusions: **Molecular urine cytology by MA and *FGFR3* mutation analysis enables a highly sensitive and specific detection of UCC. The similarity of molecular profiles in tumor and urine corroborate their clonal relation.**

INTRODUCTION

UCC[3] of the bladder is the fifth most common nonskin malignancy in the Western world with ~54,300 new cases in the United States per year (1). In most patients, UCC is superficial at first presentation. After TUR, these patients require repeated long-term surveillance, because up to 75% experiences one or more recurrences, and 15–25% will progress to invasive UCC (2–4). Cystoscopy currently remains standard practice for primary diagnosis and follow-up of UCC, but it is an uncomfortable, invasive, and expensive procedure. Although urinary cytology is the most widely used method for noninvasive detection, its application is limited by poor sensitivity, especially for low-grade UCC (5, 6). To enhance sensitivity, a wide variety of biological markers has been developed, of which the bladder tumor antigen and nuclear matrix protein 22 assays have been extensively studied. However, current data on these urinary tests do not justify their clinical integration (6, 7).

Microsatellites are tandem iterations of polymorphic di-, tri-, or tetranucleotide repeats that are found frequently throughout the genome. Microsatellite markers are altered in many cancers reflected by LOH and MSI. In UCC, the chromosomal arms 4p, 8p, 9p, 9q, 11p, and 17p often display LOH (8). In contrast with other LOHs, loss of chromosome 9 is considered an early event in bladder tumorigenesis, as it can be detected in the majority of UCCs regardless of the histopathology (8–12). In addition, it has become clear that the number of altered microsatellite markers per UCC increases with parameters of unfavorable clinical outcome (8, 9, 13).

Mao *et al.* (14, 15) and Steiner *et al.* (16) showed previously the feasibility of MA for noninvasive detection of bladder cancer in urine (14–16). Subsequently, several independent groups have confirmed the superior sensitivity of MA (75–96%) compared with morphological cytology (13–50%; Refs. 15–21). However, low-grade and low-stage tumors are still sometimes missed by MA,

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[3] The abbreviations used are: UCC, urothelial cell carcinoma; TUR, *trans*-urethral resection; LOH, loss of heterozygosity; MSI, microsatellite instability; MA, microsatellite analysis; FGFR, fibroblast growth factor receptor; TNM, Tumor-Node-Metastasis; SSCP, single-strand conformation polymorphism; AI, allelic-imbalance; CI, confidence interval.

especially if these tumors concern recurrent pTaG1 lesions, as these are usually smaller than primary cancers (16, 20).

Activating *FGFR3* gene mutations, responsible for several inherited skeletal disorders, have been detected recently in bladder cancer (22–25). Surprisingly, somatic *FGFR3* mutations in UCC are related to favorable disease with 84% of pTaG1 tumors having a mutation as compared with only 7% of ≥pT2G3 tumors (25). Therefore, the detection of these mutations in urine may provide an additional mode of noninvasive UCC detection for "favorable" UCC. To our knowledge, no report exists on the feasibility of *FGFR3* mutation detection in urine.

In the present prospective study, we explored the possibility to improve the molecular cytology diagnosis of UCC by addition of *FGFR3* mutation analysis to MA of urine. We also investigated the molecular profiles found in the tumor and the corresponding urine. In addition, we compared our molecular findings with multiple clinical variables.

PATIENTS AND METHODS

Patients. In a series of 51 patients who underwent surgery at the University Hospital Rotterdam in 1998–2000, 59 UCCs were removed in a prospective setting. Voided urine samples were obtained 1 day before surgery on admittance to the clinic. The UCCs were graded according to the WHO classification for urothelial neoplasms and staged according to the TNM classification guidelines (26, 27). In case of multifocality, the papillary lesion with the highest grade/stage was taken. The largest tumor was taken if grade/stage were the same for multiple UCCs. Four patients had one recurrent UCC and 2 patients 2 recurrent UCCs. This population consisted of 41 males and 10 females with a mean age of 64.9 years (range, 35–89) at study entrance. TUR was performed in 54, cystectomy in 4, and nephro-ureterectomy in 1 case(s), respectively. In addition, 15 consecutive control patients with a negative cystoscopical examination who were in follow-up after superficial UCC of different grades and who had a clean urinary sediment at sample collection were included. This control population consisted of 12 males and 3 females with a mean age of 65.1 years (range, 30–77) at cystoscopy. The patients signed written informed consent before study inclusion. The medical ethical committee of the Erasmus University and the University Hospital Rotterdam approved the study (MEC 168.922/1998/55). For morphological cytology, cells were stained by the standard Pap staining procedure. The final diagnosis from the cytopathology department of our hospital was used. These results were obtained on a routine basis.

Sample Collection and DNA Extraction. Freshly voided urine samples (25–100 ml) and venous blood (7 ml), as a source of reference (germ-line) DNA, were collected from each patient and stored at +4°C. Within 6 h after voiding, the urine samples were divided and processed additionally for morphological and molecular (MA and *FGFR3* status) cytology. For the molecular cytology, the urine and blood samples were handled to obtain DNA as described before (20). Standard H&E slides were made of the 59 paraffin-embedded tissue samples for the microdissection procedure. The UCC tissue was manually dissected under a microscope avoiding contamination of the DNA sample with regions of normal mucosa, leukocytes, or stroma. The samples used for the molecular analyses contained a minimum of 80% UCC cells, as assessed by histological examination. This DNA was extracted using DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). All of the DNA samples were coded and stored at −30°C until use.

***FGFR3* Mutation Analysis.** *FGFR3* mutation analysis of the various DNA samples was performed in two institutes (Josephine Nefkens Inst., Rotterdam and Inst. Curie, Paris, France) by PCR-SSCP analysis as described (24, 25). In brief, three regions encompassing activating *FGFR3* mutations found previously in severe skeletal dysplasia and UCC were amplified. The primer sequences were as reported (24). The ^{32}P-labeled PCR products were separated on 6% polyacrylamide gels in 0.2× (exon 7) or 1× SSCP buffer (exons 10 and 15; 10× SSCP buffer = 0.5 M Tris-borate and 1 mM EDTA). The PCR-SSCP procedure was checked by including the appropriate positive DNA controls and H_2O as a negative control. Samples with an aberrant band at SSCP were sequenced with T7 Sequenase v2.0 (Amersham Life Science, Inc., Cleveland, OH) to check the identity of the mutations. These analyses were carried out in a blinded fashion, without knowledge of the clinical status.

MA. In a previous study, we selected 19 microsatellite markers (ACTBP2, FGA, D16S476, D18S51, D4S243, D9S162, D9S242, D9S252, D11S488, D9S171, D16S310, THO, D9S752, D17S695, LPL, D9S144, D20S454, D17S786, and D17S960) by their performance on control DNA, informativity, and frequency of LOH in bladder cancer (20). These 19 markers are located on 12 chromosomal arms. In addition to these 19, we selected 4 microsatellite markers located within the *FGFR3* gene (4p16.3) for this study. The sequences of these 4 new primer pairs were as follows: D4S412, F = 5′-ACTACCGCCAGGCACT and *r* = 5′-CTAAGATATGAAAACCTAAGGA; D4S1614, F = 5′-CAAATGCATCATGGCACATCT and *r* = 5′-ACCATGAGCATATTTCCATTTC; D4S3034, F = 5′-CTGCCAATAAACTGGGT and *r* = 5′-TTGCTCACCAAAGAGGTT; and D4S3038, F = 5′-CTATAGGGGGGTGAAGCAACAG and *r* = 5′-TGAGAGAATATGGCTATGTGGG. The annealing temperature for these 4 additional markers was 60°C. The PCR was performed as indicated by the manufacturer of Taq-polymerase (Promega, Madison, WI). The PCR products were separated on denaturing 6% polyacrylamide-urea gels. We performed a quantitative analysis to determine the AI ratio between blood:tumor and blood:urine DNA using the PhosphorImager system and the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). LOH was scored in informative cases if a reduction of 30% in the AI ratio of the signals from tumor or urine alleles was calculated in comparison with the corresponding reference (blood) alleles. MSI was scored if a deletion or expansion of a repeat unit was found as a new band on the gel. Every microsatellite alteration (LOH and/or MSI) was confirmed by a second PCR. The AI ratio closest to 1.0 was entered into the database. The microsatellite analyses were performed in the Josephine Nefkens Institute, Rotterdam. These analyses were also done in a blinded fashion, without knowledge of the clinical status.

Statistical Analysis. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for data documentation and analysis. The χ^2 test and ANOVA (for comparison of means) were used to analyze the possible correlations between clinical variables, *FGFR3* status, and microsatellite alterations. A proportional correction for the number of noninfor-

Table 1 Correlation of clinical tumor characteristics with molecular findings in tumor tissue

	FGFR3 status			Microsatellite analysis	
	No mutation	Mutation	*P* (χ^2)	Mean number of altered markers (95% CI for mean)	*P* (ANOVA)
UCC					
Primary	14	12	=0.775	7.5 (5.9–9.0)	=0.293
Recurrent	19	14		6.3 (4.8–7.8)	
Tumor size					
<0.5 cm	11	7	=0.781	7.1 (5.3–8.9)	=0.253
0.5–2.0 cm	10	10		5.7 (3.4–7.9)	
>2.0 cm	12	9		7.7 (6.1–9.3)	
Number of tumors					
Solitary	16	22	=0.004	6.6 (5.4–7.9)	=0.664
Multiple	17	4		7.1 (5.1–9.1)	
Tumor stage					
pTa	18	26	<0.001	5.9 (4.8–7.0)	=0.002
≥pT1[a]	15	0		9.6 (7.2–12.0)	
Tumor grade					
G1	8	13	<0.001	4.3 (3.0–5.6)	=0.001
G2	10	13		7.9 (6.0–9.7)	
G3	15	0		8.8 (6.8–10.8)	
Total	33	26		6.8 (5.8–7.9)	

[a] pT1 in 3, pT2 in 8, pT3 in 2, and pT4 in 1 case(s).

mative (homozygote) cases at MA was performed when we compared the mean number of microsatellite alterations for distinct variables. Statistical significance was assumed if $P < 0.05$.

RESULTS

We have tested 59 UCC tissue samples, and the matched urine DNA with MA and *FGFR3* mutation screening. In addition, 15 control patients were included to determine the specificity of the molecular UCC diagnosis in urine.

***FGFR3* Status and Microsatellite Alterations in Tumor.** In the 59 UCCs analyzed, we found 26 (44%) mutations in the *FGFR3* gene. The mutations resulted in the amino acid changes R248C ($n = 2$), S249C ($n = 22$), and G372C ($n = 2$). No activating mutations were found in the 51 DNA samples from venous blood indicating the somatic nature of *FGFR3* mutations in UCC. In the 8 recurrent UCCs, the same mutations (S249C, three times) were detected as in the first tumor. Five recurrent cases had no mutation, which was in line with the *FGFR3* status of their previous tumor(s). Most (85%) mutations were identified in solitary pTaG1/2 UCC, whereas in none of 15 grade 3 UCCs were these mutations found (Table 1). There was no correlation between the *FGFR3* mutation and tumor size or primary/recurrent UCC (Table 1). MA of the 59 tumors revealed MSI for 7 markers and LOH for 396. Only 2 tumors had no alteration in any of the 23 microsatellite markers confirming that LOH is very frequent in UCC. There was no relation between LOH at a specific chromosomal arm and primary/recurrent UCC, tumor size, or multifocality (data not shown). Yet, the microsatellite alterations on the chromosomal arms 4p, 11q, 17p, and 20q were related to higher-stage UCC; *P*s (χ^2): 0.035, 0.035, <0.001, and 0.016, respectively, and the alterations on the chromosomal arms 4q and 17p were related to higher-grade UCC; *P*s (χ^2): 0.049 and <0.001, respectively. Twenty-eight UCCs had microsatellite alterations at 17p (14 of 15 tumors staged ≥pT1; 11 of 28 G_2 and 14 of 15 G3 UCCs). In addition,

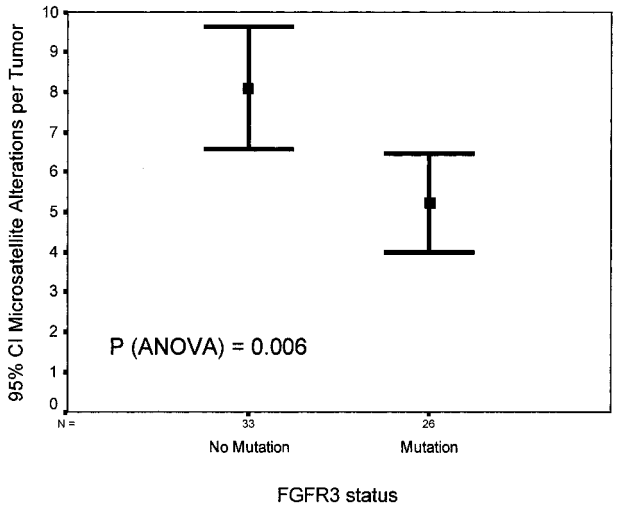

Fig. 1 Correlation *FGFR3* mutation and number of altered microsatellite markers in UCC. The figure shows the mean number of microsatellite alterations for the 33 tumors without *FGFR3* mutation, *i.e.*, 8.1 alterations/tumor (95% CI, 6.6–9.6), and for the 26 tumors with a mutation in the *FGFR3* gene, *i.e.*, 5.2 alterations/tumor (95% CI, 4.0–6.4); *bars*, CI for mean.

MA revealed significant relations between the mean number of altered markers, and tumor stage ($P = 0.002$) and grade ($P = 0.001$; Table 1). Conversely, the mean number of altered markers was significantly lower in *FGFR3* mutant tumors as indicated by Fig. 1 ($P = 0.006$). Taken together, the above-presented data provide strong evidence for the association of the *FGFR3* mutation with a more favorable kind of UCC.

***FGFR3* Mutation and LOH at 4p16.3.** We also analyzed the possible relationship between LOH in the *FGFR3* gene (chromosomal location: 4p16.3) and a mutation of *FGFR3*.

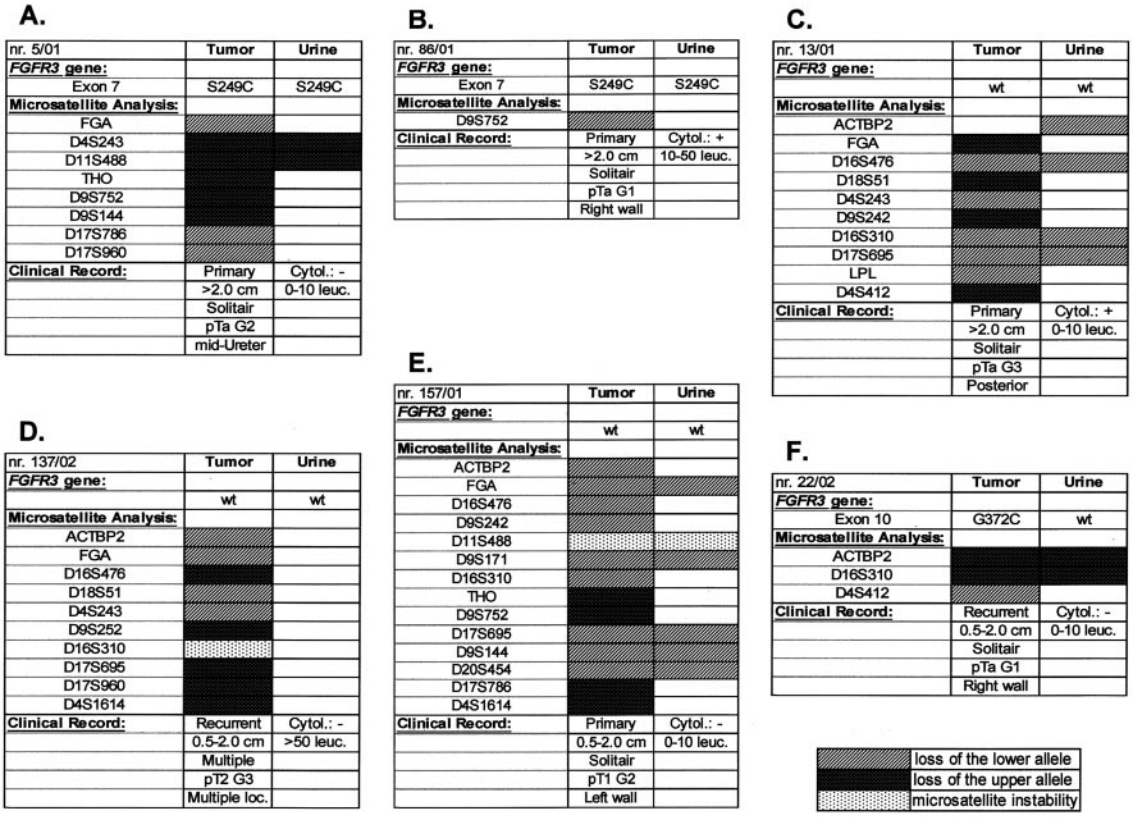

Fig. 2 Representative examples (*A–F*) of the molecular analyses on tumor and urine DNA, and the corresponding clinical records. Per case, the *FGFR3* mutations and the altered markers at MA are shown. In addition, at the bottom of each case, a clinical record is given. For the *FGFR3* status, a mutation is indicated by a *box* that contains the amino acid change caused by the mutation and the affected codon. Wild type (*wt*) indicates the absence of a mutation in the investigated exons 7, 10, and 15 of the *FGFR3* gene. For the MA, the altered markers in tumor and/or urine are presented. Loss of the upper allele, loss of the lower allele, and MSI are indicated as shown at the bottom of the figure. No LOH (AI ratio $\geq$0.70) or MSI is indicated by a *white box*. Noninformative markers are not shown. In the clinical record, information on the tumor, *i.e.,* primary or recurrent lesion, size, multifocality, pathological stage and grade, and localization, as well as information on the urine, *i.e.,* morphological cytology diagnosis and number of leukocytes per microscopic view in the urinary sediment, are given. *A* shows a *FGFR3*-positive tumor of the right upper urinary tract (*ureter*) that is detected in the corresponding urine by *FGFR3* mutation analysis and MA. *B*, a pTaG1 lesion, is not detected by MA but is detected by the *FGFR3* mutation analysis of the urine. In *C*, a LOH for the marker ACTBP2 was observed in the urine, which was not observed in the tumor. *D* shows the MA of a bladder-tumor patient with leukocyturia. Next to multiple losses, example *E* shows MSI of tumor and urine for the marker D11S488. *F* is the opposite of *B.* It shows a pTaG1 lesion that is detected by MA but not by *FGFR3* mutation analysis of urine.

Fifteen (25%) of the 59 analyzed UCCs had LOH at 4p16.3. Only 7 of these 15 also displayed a mutation in the *FGFR3* gene. As a consequence, 19 of 26 patients with a mutation had no LOH at 4p16.3. Moreover, in contrast with the *FGFR3* mutation, we found no association between a clinical variable, *i.e.,* primary/recurrent UCC, multifocality, size, stage, or grade, and LOH at 4p16.3. These data suggest that the occurrence of LOH at 4p16.3 and the *FGFR3* mutation are separate events in UCC tumorigenesis as reported recently by Sibley *et al.* (23).

Molecular and Morphological Cytology. Several representative examples of the molecular analyses on tumor and urine DNA, and the corresponding clinical records are given in Fig. 2. We investigated whether the presence of leukocytes had an impact on the outcome of our urine analyses. In 6 of the 59 urine samples, >50 leukocytes per microscopic view (magnification $\times$400) were found (example Fig. 2*D*). Both molecular (MA and *FGFR3* status) and morphological cytology detected only 1 of these 6 cases. Therefore, we excluded these 6 cases. For the remaining 53 urine samples, the sensitivity of MA, the *FGFR3* mutation, and morphological cytology were 44 of 53 (83%), 11 of 53 (21%), and 13 of 51 (25%), respectively. In this group of 53 UCCs, 21 tumors were found to have a *FGFR3* mutation. Eleven of these displayed the same mutation in the urine, whereas the 32 *FGFR3* wild-type tumors displayed no mutations in the urine. Because the sensitivity of MA was lower for UCCs with *FGFR3* mutation (15 of 21; 71%) compared with *FGFR3* wild-type UCC (29 of 32; 91%), we explored the possibility to combine MA and *FGFR3* mutation screening to enhance the sensitivity. Indeed, we detected 3 more UCCs by including the *FGFR3* status to the MA of urine. Consequently, the molecular detection of *FGFR3*-positive UCC increased from 15 of 21 to 18 of 21, and the overall sensitivity of molecular cytology increased to 47 of 53 (89%). The combination of molecular cytology and morphological cytology did not additionally enhance the sensitivity or decrease the specificity (data not shown). Table 2 shows the performance of molecular and

Table 2 Molecular and morphological urine cytology for different clinical and molecular tumor features

	Molecular cytology[a]	Morphological cytology[b]
UCC		
Primary	22/23 (95)	8/21 (30)
Recurrent	25/30 (83)	5/30 (17)
Tumor size		
<0.5 cm	14/18 (78)	2/18 (11)
0.5–2.0 cm	14/16 (88)	3/16 (19)
>2.0 cm	19/19 (100)	8/17 (47)
Number of tumors		
Solitary	30/34 (88)	4/32 (13)
Multiple	17/19 (89)	9/19 (47)
Tumor stage		
pTa	34/39 (87)	7/38 (18)
≥pT1[c]	13/14 (93)	6/13 (46)
Tumor grade		
G1	14/18 (78)	2/17 (12)
G2	20/21 (95)	4/21 (19)
G3	13/14 (93)	7/13 (54)
FGFR3 status (tumor)		
Mutation	18/21 (86)	2/20 (10)
No mutation	29/32 (91)	11/31 (36)
Microsatellite analysis (tumor)		
LOH/MSI	47/51 (92)	13/49 (27)
No LOH/MSI	0/2 (0)	0/2 (0)
Sensitivity total	47/53 (89)	13/51 (25)
Specificity total	14/15 (93)	14/15 (93)

[a] Combination of microsatellite analysis and *FGFR3* mutation detection in voided urine.
[b] In 2 cases, cytology diagnosis was not possible.
[c] See footnote Table 1.

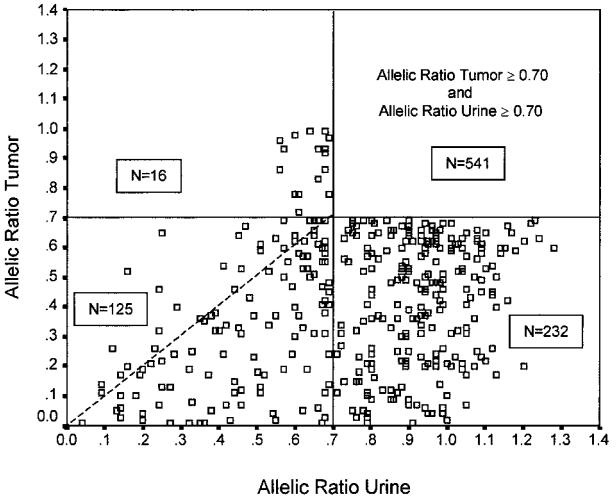

Fig. 3 Allelic (imbalance) ratios of tumor and urine for the 23 microsatellite markers. This scatterplot contains the microsatellite data of the 23 markers for the 53 UCC cases with no leukocyturia. In 305 cases, the particular microsatellite marker was not informative (homozygote). Thus, 914 informative cases remained. In 541 cases, no loss in tumor and urine was observed. Sixteen cases showed an alteration (AI ratio <0.70) in urine, which was not found in the analyzed tumor. On the other hand, 232 alterations were only detected in tumor. In 125 cases, the tumor and the urine were found to contain LOH for the same allele. For the samples below the dashed line ($n = 96$), the AI ratio was higher, *i.e.*, closer to 0.70, in urine as compared with tumor.

morphological cytology for the different clinical and molecular variables of the tumors. The superior sensitivity of the molecular detection compared with the morphological approach is evident for all of the clinical and molecular variables. Furthermore, we determined the specificity of molecular and morphological cytology on the urine samples of 15 patients who were in follow-up after TUR of superficial UCC. These patients had a negative cystoscopy and a clean (leukocytes: 0–10 per microscopic view) sediment at time of urine collection. Fourteen of these 15 had no microsatellite alterations, and *FGFR3* mutations and morphological cytology diagnosis was negative. Consequently, the specificity of molecular and morphological cytology was 93% (Table 2). In the 1 remaining patient, all three of the urine analyses were positive. A tumor obtained from the archive showed the same microsatellite alterations and the same *FGFR3* mutation as were found in the urine, suggesting that the LOHs observed in the urine were caused by a recurrence that preceded cystoscopical detection. In summary, the molecular cytology by the MA and the *FGFR3* mutation provided a highly sensitive and specific mode of noninvasive UCC detection.

Comparison of Genetic Aberrations in Tumor and Corresponding Urine. For the 53 patients without leukocyturia, we found 141 microsatellite alterations and 13 *FGFR3* mutations in the urine. In every case, at least one match between urine and tumor was found with both molecular methods. The AI ratios obtained at the MA for tumor and urine are plotted against each other in Fig. 3. In case of LOH for tumor and urine ($n = 125$), the same allele (upper or lower band on the gel) was always lost. In addition, a LOH in urine, which was not observed in the analyzed tumor, was only found in 16 of 914 (1.8%) of the informative cases (Fig. 3). These data indicate that the urine contained the same genetic aberrations as the analyzed tumor. We also observed that the AI ratios in urine were generally higher (closer to 0.70) than the AI ratios in tumor pointing to a so-called dilution effect caused by the presence of normal and malignant cells in the urine. In this light, it is not surprising that contamination of the urine by "normal" leukocytes leads to a less-sensitive detection of UCC as reported in the previous paragraph and in Table 3. In this table, we compared the molecular findings in tumor and urine for the cases without and with leukocyturia. Of the 357 microsatellite alterations in tumor, 125 (35%) were also found in the corresponding urine in case of no leukocyturia. For patients with *FGFR3* mutant tumors (33 of 100; 33%) and for patients with *FGFR3* wild-type tumors (92 of 257; 36%) microsatellite alterations were also found in the corresponding urine ($P = 0.20$). Taking into account that UCCs characterized by the presence of a *FGFR3* mutation (see also Fig. 1) are associated with a lower number of microsatellite alterations, this provides an additional explanation for the lower sensitivity of urinary MA in case of "favorable" UCC. Furthermore, we investigated whether the clinical variables had an influence on the detection of the same alterations in the urine. If multifocality was present, significantly more microsatellite (tumor) alterations were observed in urine. The numbers were 62 of 132 (47%) in case of multiple tumors and 63 of 225 (28%) in case of a solitary lesion ($P < 0.001$). In contrast, no such differences were found for the other variables, *i.e.*, primary/recurrent UCC, size, stage, or grade.

In addition to the variables mentioned above, associated carcinoma *in situ* (present in 6 cases) was also considered. The stage

Table 3 Correlation of *FGFR3* status and LOH between tumor and urine, and the effect of leucocyte abundance

A. *FGFR3* status

	Mutation (Tumor)	Same mutation (%) (Urine)	P (χ^2)
Leuc. <50 (n = 53)	21	11 (52%)	P = 0.033
Leuc. ≥50 (n = 6)	5	0 (0%)	

B. Microsatellite analysis

	Microsatellite alterations (Tumor)	Same microsatellite alteration (%) (Urine)	P (χ^2)
Leuc. <50 (n = 53)	357	125 (35%)	<0.001
Leuc. ≥50 (n = 6)	46	1 (2%)	

and grade of the papillary UCCs was as follows: pT1G2 in 1, pT1G3 in 1, and ≥pT2G3 in 4 case(s), respectively. None of these tumors had a *FGFR3* mutation, and no additional microsatellite alterations or *FGFR3* mutations in urine were seen for these cases.

DISCUSSION

Bladder cancer has a high incidence and requires continuous clinical attention after initial treatment. The urine of these patients may offer a convenient mode of noninvasive UCC detection. However, morphological cytology and other urinary tests approved by the FDA can only be used as adjuncts to cystoscopy (5–7, 20). For this reason, research efforts for noninvasive methods for detection and follow-up, which eventually could reduce the number of bothersome cystoscopical examinations, are still warranted.

The current study is to our knowledge the first to combine *FGFR3* mutation analysis and MA on matched tumor and voided urine samples. We detected 26 *FGFR3* mutations in 59 UCCs. These mutations were strongly associated with favorable (solitary pTaG1/2) UCC. The association of the *FGFR3* mutation with a lower number of LOHs per tumor and the association with solitary UCC additionally underlines the "favorable" nature of tumors with a *FGFR3* mutation found in two earlier studies (24, 25). These two recent studies described the relation of the *FGFR3* mutation with low-stage and low-grade UCC, a lower recurrence rate of superficial tumors in a series of 72 UCCs (24), and absence of *FGFR3* mutations in 20 cases of carcinoma *in situ* (the putative precursor of invasive UCC; Ref. 25). Moreover, only 4 of 28 UCCs with LOH on chromosome 17p simultaneously had a *FGFR3* mutation in the present study. As LOH at 17p (*TP53* gene locus) is indicative for high-grade and invasive UCC (Refs. 2, 9, 28; this study), this again pointed to the favorable nature of UCC with the *FGFR3* mutation.

The high incidence and the association of the *FGFR3* mutation with favorable UCC prompted us to explore its potential as a molecular marker in urine. We chose to combine the *FGFR3* mutation analysis to MA of urine because MA has proven to be a very accurate molecular method for detection and follow-up of UCC in the hands of several independent groups (14–21). Here we reported the highest sensitivities for MA in large (100%), invasive (93%), and poorly differentiated (93%) UCC. Similarly, Schneider *et al.* (18), who evaluated MA in a large group of 183 patients, also found the highest sensitivities in invasive (95%) and G3 (96%) UCC. The sensitivities for the detection of G1 UCC by MA were 78% in the present study and 79% in the study by Schneider *et al.* (18). Because we could enhance the sensitivity of molecular cytology to 89% (47 of 53) by adding the *FGFR3* mutation analysis, without compromising the specificity (93%; 14 of 15), we advocate the addition of the *FGFR3* mutation analysis, particularly the exons 7 and 10, to MA of urine to optimize the molecular cytological diagnosis for patients with low-grade UCC.

We compared the molecular profiles of our patients in UCC tissue and the corresponding urine. Interestingly, additional alterations in the urine, which were not observed in tumor tissue, were only found in 16 of 914 (1.8%) cases for MA (23 markers) and 2 of 159 (1.3%) for *FGFR3* mutation analyses (exons 7, 10, and 15). These results show that tumor cells in the urine are clonally related to the tumor(s) in the bladder. Earlier studies on smaller numbers of UCCs and their corresponding urine samples also pointed to this clonal relation (14–16). In addition, the data presented here show that significantly more microsatellite alterations reappeared in the urine if multifocality was present. Because we only analyzed 1 tumor in each case, this again provided an indication that multiple tumors are clonally related. Our results are in line with other studies that found clonal relations between multiple UCC (29, 30), upper and lower tract UCC (10, 11), and metachronous UCC (31). In the present study, 52% (11 of 21) of the *FGFR3* mutations and 35% (125 of 357) of the microsatellite alterations that were found in tumor tissue were also present in the urine. Much higher percentages (>80%) were reported in two studies using *p53* mutations as biomarkers (32, 33). This may indicate that tumor cells of UCC with p53 mutations shed more easily than *FGFR3* mutant tumor cells. However, recently developed methods for the detection of single nucleotide changes may additionally increase the sensitivity for detection of *FGFR3*-positive UCC in urine (34).

In a previous study on MA for follow-up of superficial bladder cancer (20), we found that leukocyte abundance induced false-negative results in MA. In this study, only 2% of the tumor-associated microsatellite alterations were detected in urine if leukocyturia was present. This was 35% for the patients with a clean urinary sediment. Consequently, the contamination with "normal" leukocyte DNA rendered the result unreliable. Surprisingly, Christensen *et al.* (35) reported frequent (59%) MSI in urine of patients with cystitis caused by benign prostatic hyperplasia. As a possible explanation for their discrepant outcome, Christensen *et al.* (35) pointed to the use of different (more prone to MSI) microsatellites in their study. Nevertheless, leukocyte abundance may be considered a drawback for the molecular cytology diagnosis in urine. Possibilities to circumvent this drawback were discussed before (20).

In conclusion, the clonal relation of UCC allows a detection of tumor activity in voided urine. We have shown that molecular cytology by MA and *FGFR3* gene analysis enables a highly sensitive and equally specific detection of UCC compared with morphological cytology. As molecular methods become more available with the introduction of automated techniques, our results and those of others strongly suggest that PCR-based molecular cytology may be a useful tool to improve the detection and monitoring of patients with UCC.

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