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

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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 leukocyturia. The sensitivity of MA was 125 of 357 for the 53 cases without leukocyte contamination. 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 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 tetrancleotide 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 cancers reflected by LOH, 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,
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, 30–89) at study entrance. TUR was performed in 54, cystectomy in 4, and nephro-ureterectomy in 1 case(s), respectively. In 35 males and 10 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). The UCCs were graded according to the WHO classification guidelines.

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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 32P-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 mM Tris-borate and 1 mM EDTA). The PCR-SSCP procedure was checked by including the appropriate positive DNA controls and H2O 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′-CTAAA-GATATGAAAACCTAAGGA; D4S1614, F = 5′-CAAAGT-CATCATGGCACATCT and r = 5′-ACCATGAGCATATTCTCATTTC; D4S3034, F = 5′-CTGCAATTAACCTGGGT and r = 5′-TTGTCACAACAGGTT; and D4S3038, F = 5′-CTATAAGGGGTGAAGCACAAG and r = 5′-TTGAGGAATATTGCTATGGGG. The annealing temperature for these 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 gels. We performed a quantitative analysis to determine the AI ratio between blood/tumor and blood/tumorine 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 χ2 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-
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; $Ps (x^2): 0.035, 0.035, <0.001, and 0.016$, respectively, and the alterations on the chromosomal arms 4q and 17p were related to higher-grade UCC; $Ps (x^2): 0.049 and <0.001$, respectively. Twenty-eight UCCs had microsatellite alterations at 17p (14 of 15 tumors staged $\geq$ pT1; 11 of 28 G2, and 14 of 15 G3 UCCs). In addition, 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 $FGFR3$. In the 59 tumors 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; $Ps (x^2): 0.035, 0.035, <0.001, and 0.016$, respectively, and the alterations on the chromosomal arms 4q and 17p were related to higher-grade UCC; $Ps (x^2): 0.049 and <0.001$, respectively. Twenty-eight UCCs had microsatellite alterations at 17p (14 of 15 tumors staged $\geq$ pT1; 11 of 28 G2, and 14 of 15 G3 UCCs). In addition, 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.

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Fifteen (25%) of the 59 analyzed UCCs had LOH at 4p16.3. Only 7 of these 15 also displayed a mutation in the \textit{FGFR3} gene. As a consequence, 19 of 26 patients with a mutation had no LOH at 4p16.3. Moreover, in contrast with the \textit{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 \textit{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 ×400) were found (example Fig. 2D). Both molecular (MA and \textit{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 \textit{FGFR3} mutation, and morphological cytology were 44 of 53 (83%), 11 of 53 (21%), and 13 of 53 (25%), respectively. In this group of 53 UCCs, 21 tumors were found to have a \textit{FGFR3} mutation. Eleven of these displayed the same mutation in the urine, whereas the 32 \textit{FGFR3} wild-type tumors displayed no mutations in the urine. Because the sensitivity of MA was lower for \textit{FGFR3} mutation (15 of 21; 71%) compared with \textit{FGFR3} wild-type UCC (29 of 32; 91%), we explored the possibility to combine MA and \textit{FGFR3} mutation screening to enhance the sensitivity. Indeed, we detected 3 more UCCs by including the \textit{FGFR3} status to the MA of urine. Consequently, the molecular detection of \textit{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

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Fig. 2 Representative examples (A–F) of the molecular analyses on tumor and urine DNA, and the corresponding clinical records. Per case, the \textit{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 \textit{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 \textit{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 ≥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 \textit{FGFR3}-positive tumor of the right upper urinary tract (ureter) that is detected in the corresponding urine by \textit{FGFR3} mutation analysis and MA. B, a pTaG1 lesion, is not detected by MA but is detected by the \textit{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 \textit{FGFR3} mutation analysis of urine.
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.

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

<table>
<thead>
<tr>
<th>UCC</th>
<th>Molecular cytology&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Morphological cytology&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>22/23 (95)</td>
<td>8/21 (30)</td>
</tr>
<tr>
<td>Recurrent</td>
<td>25/30 (83)</td>
<td>5/30 (17)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.5 cm</td>
<td>14/18 (78)</td>
<td>2/18 (11)</td>
</tr>
<tr>
<td>0.5–2.0 cm</td>
<td>14/16 (88)</td>
<td>3/16 (19)</td>
</tr>
<tr>
<td>&gt;2.0 cm</td>
<td>19/19 (100)</td>
<td>8/17 (47)</td>
</tr>
<tr>
<td>Number of tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solitary</td>
<td>30/34 (88)</td>
<td>4/32 (13)</td>
</tr>
<tr>
<td>Multiple</td>
<td>17/19 (89)</td>
<td>9/19 (47)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTa&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34/39 (87)</td>
<td>7/38 (18)</td>
</tr>
<tr>
<td>Mutation</td>
<td>13/14 (93)</td>
<td>6/13 (46)</td>
</tr>
<tr>
<td>FGFR3 status (tumor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No mutation</td>
<td>29/32 (91)</td>
<td>11/31 (36)</td>
</tr>
<tr>
<td>Microsatellite analysis (tumor)</td>
<td>47/51 (92)</td>
<td>13/49 (27)</td>
</tr>
<tr>
<td>LOH/MSI</td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Sensitivity total</td>
<td>47/53 (89)</td>
<td>13/51 (25)</td>
</tr>
<tr>
<td>Specificity total</td>
<td>14/15 (93)</td>
<td>14/15 (93)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Combination of microsatellite analysis and FGFR3 mutation detection in voided urine.

<sup>b</sup> In 2 cases, cytology diagnosis was not possible.

<sup>c</sup> See footnote Table 1.

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
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 non-invasive 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 because MA has proven to be 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.

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

<table>
<thead>
<tr>
<th>A. FGFR3 status</th>
<th>Mutation (Tumor)</th>
<th>Same mutation (%) (Urine)</th>
<th>P (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leuc. &lt;50 (n = 53)</td>
<td>21</td>
<td>11 (52%)</td>
<td>P = 0.033</td>
</tr>
<tr>
<td>Leuc. ≥50 (n = 6)</td>
<td>5</td>
<td>0 (0%)</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>B. Microsatellite analysis</th>
<th>Microsatellite alterations (Tumor)</th>
<th>Same microsatellite alteration (%) (Urine)</th>
<th>P (χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leuc. &lt;50 (n = 53)</td>
<td>357</td>
<td>125 (35%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leuc. ≥50 (n = 6)</td>
<td>46</td>
<td>1 (2%)</td>
<td></td>
</tr>
</tbody>
</table>

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.
REFERENCES


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

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