A Simple and Fast Method for the Simultaneous Detection of Nine Fibroblast Growth Factor Receptor 3 Mutations in Bladder Cancer and Voided Urine
Johanna M.M. van Oers, Irene Lurkin, Antonius J.A. van Exsel, Yvette Nijsen, Bas W.G. van Rhijn, Madelon N.M. van der Aa, and Ellen C. Zwarthoff

Abstract

**Purpose:** Mutations in the fibroblast growth factor receptor 3 (FGFR3) occur in 50% of primary bladder tumors. An FGFR3 mutation is associated with good prognosis, illustrated by significantly lower percentage of patients with progression and disease-specific mortality. FGFR3 mutations are especially prevalent in low grade/stage tumors, with pTa tumors harboring mutations in 85% of the cases. These tumors recur in 70% of patients. Efficient FGFR3 mutation detection for prognostic purposes and for detection of recurrences in urine is an important clinical issue. In this paper, we describe a simple assay for the simultaneous detection of nine different FGFR3 mutations.

**Experimental Design:** The assay consists of one multiplex PCR, followed by extension of primers for each mutation with a labeled dideoxynucleotide. The extended primers are separated by capillary electrophoresis, and the identity of the incorporated nucleotide indicates the presence or absence of a mutation.

**Results:** The assay was found to be more sensitive than single-strand conformation polymorphism analysis. Mutations could still be detected with an input of only 1 ng of genomic DNA and in a 20-fold excess of wild-type DNA. Moreover, in urine samples from patients with a mutant tumor, the sensitivity of mutation detection was 62%.

**Conclusions:** We have developed a fast, easy to use assay for the simultaneous detection of FGFR3 mutations, which can be of assistance in clinical decision-making and as an alternative for the follow-up of patients by invasive cystoscopy for the detection of recurrences in urine.

Bladder cancer is the fifth most common cancer in the western world with an incidence of 20 new cases per year per 100,000 people in the U.S. (1). Unfortunately, these statistics do not include superficial pTa bladder cancer, which represents the most common type of bladder cancer. In the Netherlands, the incidence of both superficial and invasive bladder cancer is estimated as about 30 new cases per year per 100,000 people. This is in accordance with data from global cancer statistics for the western world (2). Superficial bladder tumors are removed by transurethral resection. However, up to 70% of these patients will develop one or more recurrences, and it has been estimated that 1 in 1,450 people is under surveillance for bladder cancer in the United Kingdom (3). Cystoscopy is an uncomfortable, invasive, and expensive procedure, but currently remains the gold standard for detection of recurrences. Because patients have to be monitored perpetually and have a long-term survival, bladder cancer is the most expensive cancer when calculated on a per patient basis (4).

Activating mutations in the fibroblast growth factor receptor 3 (FGFR3) gene have been reported in >50% of primary bladder tumors (5, 6). Most of the somatic mutations found in bladder cancer are identical to germ line mutations responsible for skeletal disorders such as thanatophoric dysplasia and achondroplasia (7). It has been reported that FGFR3 mutations are very frequent in bladder tumors of low stage and grade, indicating that they occur much more frequently in superficial bladder cancer than in invasive bladder cancer (8, 9).

Many potential molecular markers for progressive disease have been identified (10, 11), of which Ki-67 labeling (MIB-1 staining) seemed to be the most promising marker. However, no single marker was able to predict the clinical behavior of bladder tumors, and none of the markers proved to be superior to histopathologic staging and grading (10). We recently showed that the presence or absence of an FGFR3 mutation in combination with high or low MIB-1 staining was able to characterize almost 90% of 286 primary bladder tumors (6).

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In a retrospective study with a median follow-up of 5 years, multivariate analysis showed that the combination of FGFR3 and MIB-1 seemed superior to other variables for predicting progression of bladder tumors and survival of the patients. Moreover, FGFR3 mutation analysis and MIB-1 staining were more reproducible than histochemoanalysis of the tumors by expert urinary pathologists. This was the first study to show that molecular markers can be used to predict disease course more accurately and reproducibly than traditional clinical pathology.

Over the years, the need for an inexpensive, noninvasive, and simple procedure for the detection of bladder cancer has been expressed (11, 12). Cytology done on voided urine is a noninvasive procedure with up to 100% specificity. Unfortunately, this method is limited by its sensitivity, which is especially poor for low-grade tumors. Because of this limited sensitivity, several other methods were developed for the detection of tumor cells in voided urine. Currently, one of the most promising approaches is microsatellite analysis, with a reported sensitivity of up to 95% (13, 14). Recurrences that are missed are usually small pTa grade 1 tumors. We and others have previously shown that the sensitivity of molecular cytology could be enhanced by FGFR3 gene analysis (15, 16).

Reliable and easy to perform FGFR3 mutation detection for prognostic purposes and for the detection of recurrences in urine is an important clinical issue. Therefore, a simple assay for detecting mutations is needed to replace the current labor-intensive mutation analysis by single-stranded conformation polymorphisms (SSCP) or by sequencing (16). In this study, we report the development of a new method for FGFR3 mutation analysis based on the detection of single nucleotide changes. With this method, the nine most common mutations can be detected in one assay simultaneously. In addition, it is fast and easy to use. The assay is more sensitive than SSCP, needs little DNA, and can reliably detect mutations in a background of up to 20-fold excess of control DNA.

Materials and Methods

Sample collection and DNA extraction. Paraffin-embedded tumor tissue samples were collected from patients who underwent surgery at the Erasmus MC or other hospitals in the Rotterdam area. All patients signed written informed consent. Voided urine samples were collected before surgery. DNA was obtained as previously described (14, 15).

Polymerase chain reaction. Initially, three regions of the FGFR3 gene, located in exons 7, 10, and 15, were amplified by PCR as described previously (8). These regions comprise the following potential codon mutations: R248C and S249C (exon 7), G372C, Y375C, and A393E (exon 10), and K652E, K652Q, K652M, and K652T (exon 15).

After analysis of the secondary structure and folding free energy of the exon 7 and exon 10 PCR products using DNAMAN software (Lynnon Corporation, Vaudreuil, Canada), new primer sequences were constructed. In the end, the following primer pairs were used: for exon 7, 5'-AGTGGCGGTGGTGTTGAAGCAG-3' and 5'-GCACCGGC- GTCTGGTGTC-3'; for exon 10, 5'-CAAAGGCGCACTGTTGACAG-3' and 5'-AGGCCAGACGGTACAG-3'; and for exon 15, 5'-GACGGAGGCA- CAACGGTGATG-3' and 5'-GTGTGGAGGGCGCTGGTCG-3'. A multiplex reaction for all three regions was successfully set up. The multiplex PCR was done in a volume of 15 μL, containing 1× PCR buffer, 1.5 mmol/L MgCl₂, 0.5 units Taq polymerase (Promega, Madison, WI), 0.17 mmol/L dNTPs, and 1 to 250 ng of genomic DNA. Cycling conditions were as follows: 5 minutes at 95°C, 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by 10 minutes at 72°C. Products were treated with Exonuclease I (ExoI, Amersham Biosciences, Uppsala, Sweden) and shrimp alkaline phosphatase (Amersham Biosciences) to remove excess primers and dideoxyribonucleotide triphosphates.

Fibroblast growth factor receptor 3 mutation analysis. PCR products were analyzed for mutations using the ABI PRISM SNPShot Multiplex Kit (Applied Biosystems, Foster City, CA), according to the protocol supplied by the manufacturer. The SNPShot method is based on the dyeoxy single-base extension of unlabeled oligonucleotide primers. For each of the nine mutations described above, a primer annealing adjacent to the potentially mutant nucleotide was developed. In mutations K652E and K652Q, the same nucleotide is changed, therefore, one primer will detect both mutations. The same holds for mutations K652M and K652T. In total, seven SNPShot primers were developed, with a different length of poly(dT) tail attached to the 5'-end to enable their simultaneous detection. All primers were designed with a similar melting temperature and were checked for the absence of base pairing with other SNPShot primers.

PCR conditions were optimized in order to develop a multiplex SNPShot reaction. Two new primers had to be developed to improve detection of the S249C and Y375C mutations (see Results). In order to enhance the detection of the most frequent mutation (S249C), two different lengths of poly(dT) tails were added. The final panel of SNPShot primers is shown in Table 1. The multiplex SNPShot reaction was done in a volume of 10 μL, containing 2 μL of PCR product (see above), 2.5 μL Ready Reaction Mix, 1× sequencing buffer, and SNPShot primers with concentrations as indicated in Table 1. Cycling conditions were, 25 cycles of rapid thermal ramp to 96°C, 96°C.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Strand</th>
<th>Primer extension</th>
<th>Concentration (pmol/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R248C</td>
<td>5'-T49CGTCTACGCCCCACAGG-3'</td>
<td>sense</td>
<td>C</td>
<td>2.0</td>
</tr>
<tr>
<td>S249C</td>
<td>5'-T34TCTGCCCCCACAGGGGCT-3'</td>
<td>sense</td>
<td>C</td>
<td>1.2</td>
</tr>
<tr>
<td>S249C</td>
<td>5'-T33TCTGCCACACAGGCGCT-3'</td>
<td>sense</td>
<td>C</td>
<td>1.2</td>
</tr>
<tr>
<td>G372C</td>
<td>5'-T35GTTGGAGGCTGACAGGCCG-3'</td>
<td>sense</td>
<td>G</td>
<td>0.4</td>
</tr>
<tr>
<td>Y375C</td>
<td>5'-T37AGAGGGCGGACGTGGT-3'</td>
<td>sense</td>
<td>A</td>
<td>0.6</td>
</tr>
<tr>
<td>A393E</td>
<td>5'-T33GCTGTCTACGCCATGTTGTTGG-3'</td>
<td>sense</td>
<td>C</td>
<td>2.4</td>
</tr>
<tr>
<td>K652E/Q</td>
<td>5'-T30GCACAACCTCGACTACTACAG-3'</td>
<td>sense</td>
<td>A/G</td>
<td>1.2</td>
</tr>
<tr>
<td>K652M/T</td>
<td>5'-T20CACAACCTCGACTACTACAGA-3'</td>
<td>sense</td>
<td>A/T/C</td>
<td>0.8</td>
</tr>
</tbody>
</table>
for 10 seconds, rapid thermal ramp to 58.5°C, and 58.5°C for 40 seconds. After treatment with shrimp alkaline phosphatase to remove excess dideoxynucleotide triphosphates, labeled products were separated in a 25-minute run on 36-cm-long capillaries in an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). GeneScan Analysis Software version 3.7 (Applied Biosystems) was used for data analysis.

Results

Development of a fibroblast growth factor receptor 3 mutation detection assay. Mutation analysis was based on the ABI PRISM SNaPshot Multiplex Kit. We first designed primers adjacent to the seven nucleotides that are most frequently mutated in bladder tumors. These seven primers enabled the
detection of nine mutations in the \textit{FGFR3} gene. They were modified by adding different lengths of poly(dT) tails to the 5’-end in order to allow their separation based on differences in size. These primers were annealed to a combination of \textit{FGFR3} exon 7, 10, and 15 PCR products from control DNA samples. In a subsequent reaction (SNaPshot reaction), the primers were extended using a substrate mixture of all four fluorescently labeled dideoxynucleotide triphosphates, thus allowing the addition of only one nucleotide to each primer. The products were analyzed on an ABI automatic sequencer in a 25-minute run. With this procedure, all seven wild-type nucleotides were detected as shown in Fig. 1A.

When we subsequently analyzed known mutations in bladder tumor DNA samples, it seemed that the mutations in codons 248, 372, 393, and 652 could be detected reliably. However, for codon 249, the mutant peak was often too small to be distinguished from background signals, thus leading to false-negative results. Conversely, false-positive results were frequently experienced for codon 375 because of the presence of a background peak with the same color and position as expected for the mutant nucleotide. A new SNaPshot primer for codon 375 was therefore designed that annealed to the antisense strand. When using this forward primer, the background signal disappeared. A new primer on the opposite strand was also developed for codon 249. Two different lengths of poly(dT) tails were added to the new S249C forward primer, in order to enhance the possible detection of this most frequent mutation. However, the detection of the S249C signal continued to be inefficient. We next analyzed the secondary structure of the exon 7 PCR product, and observed that the single strand was able to fold into a very stable secondary structure with a free energy of $-36.11 \text{ kcal/mol}$. When the codon 249 mutation was present, the structure was even more stable (free energy $-40.05 \text{ kcal/mol}$). Consequently, annealing of the S249C SNaPshot primer was inefficient. A new exon 7 reverse primer was developed to create a shorter PCR product with a lower GC content, resulting in a less stable secondary structure for both wild-type (free energy $-20.31 \text{ kcal/mol}$) and codon 249 mutant (free energy $-22.88 \text{ kcal/mol}$) DNA. As a result, we now observed that the S249C peaks were clearly visible next to the wild-type peaks (detection of the codon 249 mutation is depicted in Fig. 1C). Please note that the mutant peaks appear at a slightly different position than the wild-type products. This is due to the differences in size of the fluorescent labels of the dideoxynucleotide triphosphates. In addition, the assay is not quantitative because the labels also have different emission efficiencies.

Based on the secondary structure, a new reverse primer was also developed for exon 10, and this improved the detection of the codon 375 mutation even further (Fig. 1E). In Fig. 1B-J, examples of the detection of all nine mutations are shown.

\textbf{Sensitivity of the fibroblast growth factor receptor 3 SNaPshot assay.} We next determined the minimal amount of DNA required for reliable mutation detection. To this end, tumor DNA samples harboring the four most common mutations (S249C, Y375C, R248C, and G372C) were diluted, and for each of these mutations, we observed that 1 ng of DNA was sufficient as a starting concentration for the multiplex PCR reaction (data not shown). We subsequently determined whether these mutations could also be detected in a background of normal DNA. To this end, we mixed tumor DNA heterozygous for the mutations (\textit{FGFR3} mutations are usually heterozygous; refs. 15, 17) with control DNA in different ratios (1:1, 1:4, 1:9, and 1:19). Figure 2 shows the result of the mutation detection using a 1:9 (G372C and Y375C) or 1:19 (R248C and S249C) ratio of mutant versus normal DNA. All four mutations were detected. This means that one mutant allele can still be detected against a background of up to at least 39 wild-type alleles.

\textbf{Fibroblast growth factor receptor 3 SNaPshot mutation detection is superior to single-stranded conformation polymorphisms.} To compare the new method with analysis by SSCP, we did \textit{FGFR3} mutation analysis on primary tumor DNA.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{DNA samples from tumors harboring the most common \textit{FGFR3} mutations were diluted at a ratio of 1:9 (G372C and Y375C) or 1:19 (R248C and S249C) with control (nonmutant) DNA. \textbf{A}, R248C; \textbf{B}, S249C; \textbf{C}, G372C; \textbf{D}, Y375C.}
\end{figure}
The frequency of FGFR3 mutations in primary bladder tumors is ~50%. Because the presence of an FGFR3 mutation is associated with a favorable disease course (6), a first analysis of tumors for mutations can select this 50% of patients with a low chance of progressive disease, which could reduce the number of cytoscopies. FGFR3 also has potential as a molecular marker in urine. A previous study by Rieger-Christ et al. (16) showed an overall frequency of FGFR3 mutations in urine DNA samples of 43%. In this work, the mutation status of the corresponding tumors was not analyzed, therefore it is not possible to calculate the sensitivity of FGFR3 mutation detection. We have shown that FGFR3 mutation detection on urine by the SNaPshot assay is an efficient method to detect recurrent bladder tumors (i.e., the sensitivity of the assay was 62%). That the mutations in the urine DNA samples were not observed in all cases might in part be due to the fact that FGFR3 mutant tumors are often pTaG1, and it is possible that these tumors shed fewer cells than tumors containing a wild-type FGFR3 gene, as these are more often of higher grade and stage. In three cases, the mutation in the urinary DNA sample did not match the mutation in the tumor, and in four tumors that were wild-type for FGFR3, the corresponding urine contained an FGFR3 mutation. A possible explanation for these findings is that we have observed in another study that there is a small group of patients in whom wild-type and mutant bladder tumors seem to occur simultaneously, and that some tumors also appear heterogeneous for the FGFR3 mutation. For follow-up of patients with superficial bladder cancer (pTa, pT1), we suggest the following scheme: cystoscopy is done at 3 months after transurethral resection and subsequently once per year. FGFR3 mutation analysis on urine DNA is done every 3 months. When positive, cystoscopy follows. When negative, further screening is done by loss of heterozygosity analysis. Thus, FGFR3 screening on urine DNA is combined into one assay, thus allowing a sample to be screened for all FGFR3 mutations simultaneously. Furthermore, there is no need for additional sequencing because both techniques are identical.

In conclusion, we developed a high-throughput method for FGFR3 mutation analysis that is more sensitive than SSCP, and

### Table 2. Comparison of FGFR3 SNaPshot mutation analysis with SSCP on 92 primary bladder tumor DNA samples

<table>
<thead>
<tr>
<th>FGFR3</th>
<th>SSCP</th>
<th>SNaPshot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td>R248C</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>S249C</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>G372C</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Y375C</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>A393E</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K652E</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K652M</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K652Q</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>K652T</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>92</td>
<td>92</td>
</tr>
</tbody>
</table>

2 N.M. van der Aa, unpublished results.

### Table 3. Sensitivity of FGFR3 mutation detection by SNaPshot analysis in urine samples

<table>
<thead>
<tr>
<th></th>
<th>Urine (mt)</th>
<th>Urine (wt)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (mt)</td>
<td>18*</td>
<td>11</td>
<td>29</td>
</tr>
<tr>
<td>Tumor (wt)</td>
<td>4</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>42</td>
<td>64</td>
</tr>
</tbody>
</table>

Abbreviations: mt, FGFR3 mutation present; wt, wild-type FGFR3 gene.

*In three cases, the mutation found in urine differed from the mutation in the tumor.
that can be used on both tumor and urine DNA samples. In theory, it is possible to add additional primers to the SNaPshot reaction; for instance, for the detection of other less common mutations such as G382C and S373C (7, 16, 18).

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References

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