TP53 and *FGFR3* Gene Mutation Assessment in Urine: Pilot Study for Bladder Cancer Diagnosis

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Abstract. *Aim: To assess, in a prospective clinical research study, a new non-invasive and reliable test to accurately detect tumor protein 53 (TP53) and fibroblast growth factor receptor-3 (FGFR3) mutations in cells in urine. Materials and Methods: TP53 mutations were analyzed using the functional analysis of separated allele in yeast (FASAY) method, which allows functional analysis of the P53 protein, and FGFR3 mutations were assessed with the SNaPshot system, detecting the eight most frequent point-mutations of this gene. Chi-square test or Fisher's exact test were used to compare TP53 and FGFR3 mutations in the tumors according to tumor stage and grade. Results: TP53 and FGFR3 mutations in bladder tumors increased and decreased respectively with increasing tumor stage and cellular grade ($p<0.05$ and $p<0.001$, respectively). A total of 103 tumor/urinary sediment couples were analyzed. TP53 or FGFR3 mutations were observed in 76 tumors. The sensitivity for the detection of this type of mutation in urine was 46%, the specificity was 81%, the positive predictive value was 94% and the negative predictive value was 37%. Conclusion: Our original data confirmed the feasibility of TP53 and FGFR3 mutation detection in urine sediment. These measurements, together with urine cytology, may increase tumor detection. The sensitivity of the TP53/FGFR3 phenotype test in the urine was less than 50% and was not able to replace standard cystoscopy in the diagnosis of bladder tumors.*

Bladder cancer is ranked as the 11th most common type of cancer in the world and most cases occur in developed countries, with two identified risk factors: smoking habits and occupational exposure (1, 2). Tumor stage and tumor grade are considered major prognostic factors (3). Various molecular biological studies have suggested two major pathways in bladder carcinogenesis: one for invasive tumors with a high risk of malignancy, which is characterized by the presence of mutations of the *TP53* tumor-suppressor gene (short arm of chromosome 17), and another for non-invasive tumors with low risk of malignancy, characterized by mutations in the fibroblast growth factor receptor-3 (*FGFR3*) gene (short arm of chromosome 4) (4, 5). In bladder cancer, *TP53* mutation has been found in 50-60% of muscle-invasive tumors (MIBC) or high risk of malignancy (carcinoma *in situ* (CIS), and high-grade (HG) tumors), associated with an increased rate of tumor recurrence and risk of muscle infiltration (6). *FGFR3* gene mutation has been found in 60 to 70% of non-MIBC (Ta, T1) or less aggressive tumors (7, 8). *FGFR3*-mutated tumors have a lower recurrence rate than non-mutated tumors (9). Although a number of different genetic changes contribute to bladder cancer development and progression as recently reported in the Cancer Genome Atlas Network (10), nevertheless mutations of *FGFR3* and *TP53* genes are the genetic alterations most frequently associated with bladder tumor development (11).

Currently, monitoring of patients with a previous history of bladder cancer is based on cystoscopy and urine cytology. This follow-up may be improved by using less sensitive urine cytology for the detection of low-grade tumors, in order to avoid the direct and indirect costs of repeated surveillance cystoscopy. Numerous urine tests have been proposed by pharmaceutical companies over the past decade but they were not specific or sensitive enough to be recommended for routine clinical practice (12).

The main objective of our prospective study was to develop a new non-invasive and reliable test to accurately detect *TP53* and *FGFR3* mutations in cells in the urine.

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Materials and Methods

Sample collection of urine and tumors. Urine specimens and tumoral tissues were collected from 103 patients with newly-diagnosed bladder cancer subjected to transurethral resection of the bladder between January 2011 and January 2013. Urine samples were collected from naturally voided urine before the resection and stored at 4˚C in lysis buffer solution to stabilize mRNA (13, 14). The urine was then centrifuged for 15 min at 5000 rpm at 4˚C and then at 10,000 × *g* for 10 min at 4˚C. The pellet was re-suspended with 150 μl of the supernatant and the tube was kept at −80˚C until analysis. It is important to note the distribution of high-grade and -stage bladder tumors in our series (46% of the samples), suggesting that our clinical source is more likely a referral center as observed in many University Hospitals. In order to validate our assay, controls were performed previously on urine of five patients with benign prostate hyperplasia.

TP53 mutation analysis. Extraction of mRNA, required for TP53 mutation detection in bladder tumor and urine, was carried out based on the protocol of the illustra QuickPrep Micro mRNA Purification Kit (GE Healthcare, Little Chalfont, United Kingdom). After centrifugation at 11,000 × *g* for 1 min, the supernatant containing genomic DNA was stored at −20˚C and mRNA fixed on the resin was stored at −80˚C after elution. cDNAs were then obtained by reverse transcription (RT) using a Verso Kit® (Thermo Scientific, Ilkirch, France). Amplification of the TP53 coding sequence was performed by PCR using a high-fidelity polymerase (PrimeStar® from TaKaRa, Kyoto, Japan) with the following primers: sense P3, 5'-ATTTGATGCTGTCCCCGGACG ATATTG AA-3' and anti-sense P4, 5'-ACCCTTTTTGGACTTCAGGT GGCTGGAGT-3' (15). The temperature cycles were 5 s at 94˚C, 45 cycles of 10 s at 65˚C followed by 3 min at 74˚C using 5 μl of RT product. The PCR product was subjected to electrophoresis on a 1.5% agarose gel in order to determine the amplification of a 1.2 kb fragment of the human *TP53* coding gene (Figure 1A).

Using the Functional Analysis of Separated Allele in Yeast (FASAY), a p53-functional assay developed in *Saccharomyces cerevisiae* yeast, *TP53* mutations were detected in tumoral tissues and urinary cells. For functional analysis of *TP53* protein status, yeast was cultivated in synthetic medium containing 10 mg/l of adenine in which colonies expressing wild-type functional p53 protein are white and colonies not expressing functional p53 protein (mutated *p53*) are red because of accumulation of an intermediate in adenine synthesis. A percentage of red colonies >10% indicates the presence of non-functional alleles in the tumors or in the urinary cells (15-17). In the present study, the MRC5 cell line derived from normal human cells was used as a negative control, giving rise to 10% or less of red colonies. Positive control was obtained by transforming the yeast cells with the yeast expression vector used for gap repair without the 1.2 kb *TP53* open reading frame (100% of red colonies).

FGFR3 mutation analysis. Genomic DNA extraction from tumors and urine was performed using a commercial kit (QIAamp Viral RNA® Mini kit (Qiagen, Venlo, Limbourg, The Netherlands)). *FGFR3* mutations were detected by SNaPshot (SNaPshot® Multiplex kit from Applied Biosystems, Waltham, Massachussetts, USA). Van Oers *et al*. described this simple method for finding the eight most frequent mutations of the *FGFR3* gene, located in exons 7, 10 and 15 (hotspot mutations) (18). Amplification of these three

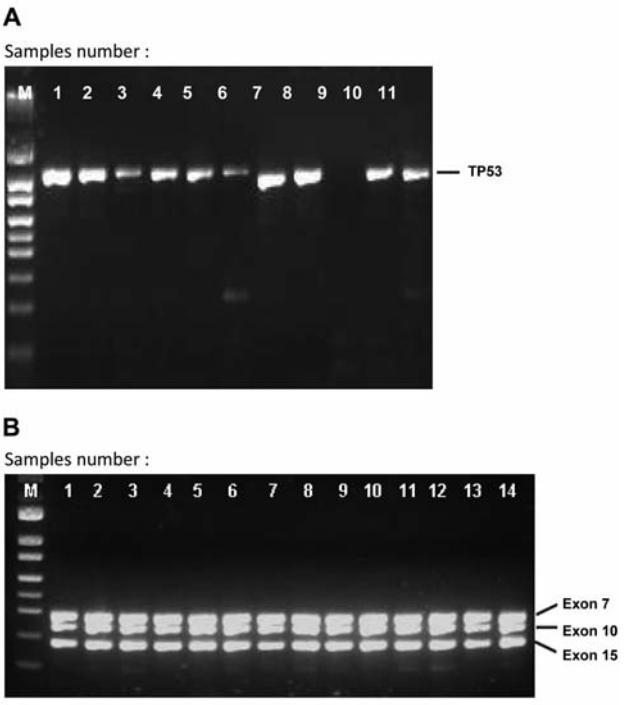

Figure 1. *Electrophoresis gel, confirming the amplification of DNA after polymerase chain reaction. A: TP53 cDNA (1.2 kb) on a 1.5% agarose gel. B: Three exons of specific interest for fibroblast growth factor receptor (FGFR3): exon 15 (266 kb), exon 10 (331 kb) and exon 7 (374 kb) on a 3% agarose gel. M: 100 bp ladder from Ozyme.*

exons was performed by PCR using a Multiplex PCR Kit (Qiagen, Venlo, Limbourg, The Netherlands) with 2.5 μl of genomic DNA (45 cycles of 90 s at 64˚C) and using the following primers: Exon 7 sense, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and anti-sense, 5'-CCCACAGCTTCTGCCCCCGA-3'; exon 10 sense, 5'-GGGCA TCCATGGGAGCC-3' and anti-sense, 5'-CAGCGTGGGCCG AGGT-3'; exon 15 sense, 5'-CCCTGAGATGCTGGGAGCAG-3' and anti-sense, 5'-GTGTGGGAAGGCGGTGTTG-3' (18). PCR products were electrophoresed on a 3% agarose gel in order to confirm the amplification of a 266 kb fragment (exon 15), a 331 kb fragment (exon 10) and a 374 kb (exon 7) (Figure 1B).

PCR product purification was performed using Shrimp Alkaline Phosphonuclease (*SAP*) and exonuclease 1 (*EXO1*) (Ozyme, Montigny le Bretonneux, France) for 1 h at 37˚C before denaturation at 75˚C for 15 min. The SnaPshot extension reaction was performed using a primer mix, hybridizing the seven common potential upstream mutation sites. After SAP purification, 1.5 μl of extension product was added to 10 μl of a mixture of formamide containing 4% of a size marker 120 LIZ® from Applied Biosystems (18) and sequenced using a 3130xl Genetic Analyzer® from Applied Biosystems in order to determine the nucleotide incorporated after the primer. Sequencing comparison of incorporated nucleotide in the sample *versus* a non-mutated MRC-5 cell-negative control defined the existence or not of mutations in tumoral tissue and urinary cells. Due to a non-practicable positive control with the eight different hotspot mutations of the *FGFR3* gene, identified

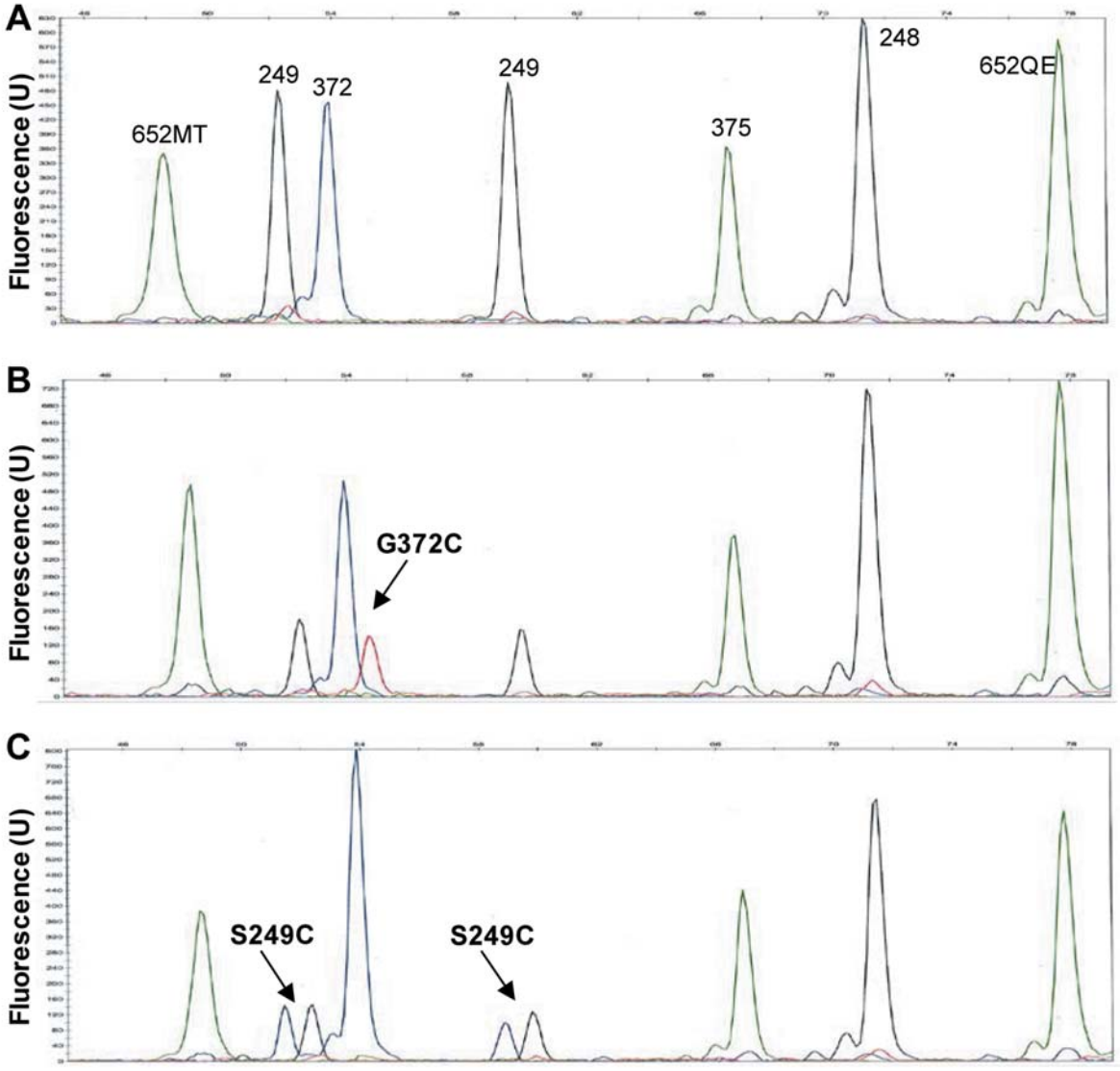

Figure 2. *Detection of wild-type and mutant fibroblast growth factor-3 (FGFR3) nucleotides using the FGFR3 SNaPshot mutation assay. A: Analysis of control DNA. Peaks are labeled with the relevant FGFR3 codon. Two different primers were used for codon 249. B and C: Analysis of DNA samples containing the G372C and S249C mutation, respectively.*

mutations in samples were confirmed by a second independent SNaPshot analysis (Figure 2) (6).

Statistical analysis. A Chi-square test was used for statistical analysis to compare *TP53* and *FGFR3* mutations in the tumors by tumor stage and grade cell. When conditions were not met for using this test, a Fisher's exact test was performed. A value of $p<0.05$ was considered significant for statistical analysis. For the analysis of the correlation between *TP53* or *FGFR3* mutations in tumors and urinary cells, sensitivity, specificity and positive and negative predictive values were determined. All of the statistical tests were performed using SPSS software®, version 17 (SPSS, Chicago, IL, USA).

Results

Mutations of *TP53* and *FGFR3* genes were analyzed in 103 primary bladder tumors and associated urine samples. Histological examination allowed for identification of 77 non-MIBC: 45 tumors were classified as Ta (44%), 21 as T1 (20%), and 11 as CIS (11%); and 26 MIBC (25.2%). A total of 29 tumors were low-grade (28%), whereas 74 were high-grade (72%), according to the WHO classification (19). (Tables I and II).

Table I. *Distribution of TP53 and fibroblast growth factor receptor 3 (FGFR3) mutations in tumors according to tumor stage.*

	TP53		*FGFR3*					
Type	WT	M	WT	M, codon 248	M, codon 249	M, codon 372	M, codon 375	M, codon 652
Ta	27 (60%)	18 (40%)	20 (45%)	6 (13%)	10 (22%)	0	8 (18%)	1 (2%)
T1	10 (48%)	11 (52%)	15 (71%)	1 (5%)	3 (14%)	0	2 (10%)	0
T2	5 (20%)	21 (80%)	21 (80%)	0	3 (12%)	1 (4%)	1 (4%)	0
CIS	5 (45%)	6 (55%)	10 (91%)	0	1 (9%)	0	0	0

CIS: Carcinoma *in situ*; WT: wild-type; M: mutated.

Table II. *Distribution of TP53 and fibroblast growth factor receptor 3 (FGFR3) mutations according to the cellular grade of the tumor.*

	TP53		*FGFR3*					
Grade	WT	M	WT	M, codon 248	M, codon 249	M, codon 372	M, codon 375	M, codon 652
Low	19 (66%)	10 (34%)	11 (38%)	4 (14%)	7 (24%)	0	7 (24%)	0
High	28 (38%)	46 (62%)	55 (74%)	3 (4%)	10 (14%)	1 (1.5%)	4 (5%)	1 (1.5%)

WT: Wild-type; M: mutated.

TP53 mutation analysis. Functional TP53 mutations were observed in 56 out of the 103 analyzed tumors (54% of cases). Analysis of *TP53* mutation occurrence according to tumor stage showed that 18 Ta tumors (40% of Ta tumors), 11 T1 (52%), 21 T2 (80%) and 6 CIS (55%) presented a mutation of the *TP53* gene. A significant difference was observed in the distribution of *TP53* mutations based on tumor stage (p=0.005), suggesting a potential prognostic value. Moreover, a significant difference in the distribution of TP53 mutations according to cellular grade (p<0.001) was also observed. Ten low-grade tumors (34% of cases) *versus* 46 high-grade tumors (62% of cases) presented a TP53 mutation.

As stated previously, a *TP53* mutation was detected in 56 tumors, whereas this type of mutation was reported in 19 corresponding samples of cells in urine (34% of cases) and not found in 37 (66% of cases). In 47 tumors, there was no *TP53* mutation and this was confirmed in the corresponding urinary sediment in 41 cases. Regarding sensitivity, the probability of detecting a *TP53* mutation in the urinary sediment confirming that in the tumor was 34% for our urine test. In contrast, the specificity was 87%, with a positive predictive value of 76% and a negative predictive value of 53%.

FGFR3 mutation analysis. FGFR3 mutations were diagnosed in 37 out of the 103 analyzed tumors (36% of cases). In 25 Ta tumors (55% of cases), six T1 (29%), five T2 (19%) and one CIS (10%), an *FGFR3* mutation was identified. We reported a negative association in the distribution of *FGFR3* mutations according to tumor stage (p=0.002). Analysis of the occurrence of *FGFR3* mutations with respect to tumor grade revealed the presence of a *FGFR3* mutation in 18 low-grade tumors (62% of the cases) and in only 19 high-grade tumors (26% of the cases). As above, a negative association in the distribution of *FGFR3* mutations according to cellular grade was identified (p<0.001). Our assessment of *FGFR3* mutations in tumors and corresponding cells in urine showed that among 37 tumors displaying a *FGFR3* mutation, 16 exhibited a corresponding mutation in cells in urine (43% of cases), whereas 21 did not (57% of patients). In 66 tumors, no *FGFR3* mutation was found, confirming 65 of the corresponding samples of cells in urine. The sensitivity was 43% and the specificity was 98%. The positive predictive value was 94%, whereas the negative predictive value was 76%.

Correlation of TP53/FGFR3 phenotype in tumors and corresponding urine sediment. In our cohort of 103 patients, 76 tumors were characterized by a *TP53* or *FGFR3* mutation (74% of cases). The sensitivity was approximately 46%. In contrast, the specificity, which was the probability of finding wild-type phenotype in the urine sample when the corresponding tumor was wild-type, was 81%. The positive predictive value was 94% and the negative predictive value was 37%.

Discussion

As part of The Cancer Genome Atlas project, Weinstein *et al*. (10) reported an integrated analysis of 131 urothelial carcinomas to provide a landscape of molecular alterations. There were statistically significant recurrent mutations in 32 genes, including multiple genes involved in cell-cycle regulation, chromatin regulation and kinase signaling pathways. In contrast, Smal *et al*. (11) recently confirmed the alternative role of *FGFR3* and *TP53* mutations in development of bladder cancer. Together, these two genetic markers were found in 62% of the studied tumors. In the present study, we identified a mutation of the *TP53* gene in 54% of the 103 bladder tumors studied, confirming its implication in bladder carcinogenesis, as suggested by Spruck *et al*. (20) and confirmed more recently by Bakkar *et al*. (4) and van Rhijn *et al*. (5). In our study, most tumors presented invasion of the lamina propria or muscle infiltration, and we recorded more *TP53* mutations for T2 than Ta tumors (80% *versus* 40%, respectively). Similarly, regarding tumor grade, a mutation was detected in 34% of low-grade tumors *versus* 62% for high-grade, underlining the implication of *TP53* in the development of aggressive tumors.

In 2006, Lamy *et al*. published preliminary results pointing to usefulness of the *TP53*/*FGFR3* genotype, assessed using the FASAY to identify *TP53* mutations (6). Similar conclusions were drawn by van Rhijn *et al*. who used immunohistochemistry for *TP53* detection (5). Recently, Neuzillet *et al*. reported interesting data based on a meta-analysis including eight relevant research studies on *TP53* and *FGFR3* mutations in bladder tumors (21). The FASAY test was used in two studies while the other authors used a sequencing method. A total of 917 tumors displaying a *TP53* mutation were analyzed and the results showed that the frequency of *TP53* mutation significantly increased with tumor stage and grade. The FASAY method to analyze the functionality of the p53 protein seems to be more relevant. Watanabe *et al*. referred not only to its superiority compared to immunohistochemistry, but also to its simplicity in routine practice in contrast to sequencing. However, it requires perfect preservation of tumoral tissue, as well as cells in urine to avoid degradation of mRNA (22).

To date, few studies have investigated *TP53* mutations in cells in urine. Eissa *et al*. assessed 100 patients with bladder tumors, 93 with benign urological disease and 47 healthy controls. They reported a sensitivity of 37% and a specificity of 100% for TP53 mutation detection in urine using the sequencing method (23). One of our concerns was whether the urinary tests reflected tumor expression. Prescott *et al*. evaluated *TP53* mutations in tumors and corresponding urine sediment of 49 patients, with a sensitivity of 84% and a specificity of 97% (24). Schlichtholz *et al*., who used the FASAY test, also suggested the prognostic value of *TP53* mutations (25). The results of our prospective series demonstrates the efficacy of FASAY, with a mutation rate of 50% in tumors, even if the sensitivity of the urine test for *TP53* mutation detection was low, as a *TP53* mutation rate of only 30% was found in urine sediment. These factors may probably explain the low sensitivity observed, since there was an insufficient number of analyzed tumor cells in urine sediment, and this could have hindered the detection rate of *TP53* mutations. In FASAY, samples containing wild-type *TP53* commonly produce a background of 5-10% of red colonies due to PCR-induced errors and the presence of an alternatively spliced *TP53* mRNA. In tumors, more than 10% red colonies revealed the existence of *TP53* mutation with both alleles inactivated when the percentage was >50%. This rate of 10% in order to define mutated p53 cells in urine, based on studies of 1995 and 1998, could be debated (15).

Concerning the *FGFR3* mutations in bladder tumors and cells in urine samples, we recorded this mutation for 37 patients (36%) in our previously described cohort of patients. In contrast to *TP53, FGFR3* mutations were more frequently identified in low-grade tumors (62% *versus* 26% in high-grade tumors), and non-invasive tumors (55% of Ta, 29% of T1 and 19% of T2). Lamy *et al*. reported the usefulness of the SNaPshot for *FGFR3* mutation analysis. A negative correlation of *FGFR3* mutation to tumor stage and cell grade was observed (6). Van Rhijn *et al*. described a similar distribution of *FGFR3* mutation according to tumor stage and grade using the sequencing technique (5). Finally, in meta-analysis by Neuzillet *et al*., *FGFR3* mutation detection was performed using sequencing or SNaPshot with equivalent data: 65% of Ta and 70% of G1 tumors in contrast to 11% of T2 and 19% of G3 tumors (20).

For the detection of *FGFR3* mutation in cells in urine, we found that the sensitivity of the SNaPshot test was 43%. Specificity was excellent at 98% and the positive predictive value was 94% and negative predictive value 76%. These data were significantly higher than those reported for the detection of *TP53* mutation, although the sensitivity was lower. Van Rhijn *et al*. reported their findings regarding *FGFR3* mutation sequencing, where 59 bladder tumors and corresponding urinary sediment were analyzed (5). For the 21 patients where a *FGFR3* mutation was observed in bladder tumor, the same mutation was only identified in the urine sediment of 11 patients. The sensitivity of the urine test was 52%, with a specificity of 100%, similar to our experience. Van Oers *et al*. described SNaPshot results for 64 bladder tumors and corresponding urine sediment and suggested the usefulness and efficacy of this method: 29 tumors had an *FGFR3* mutation which was found in 18 corresponding urine sediment samples (sensitivity of 62% with a specificity of 88%) (18).

Finally, as previously reported in the literature, our results underlined the "mirror distribution" of *TP53* and *FGFR3* mutations, which were strongly correlated to tumor stage and grade. Moreover, we validated the FASAY and the SNaPshot techniques for the detection of *TP53* and *FGFR3* mutations, not only in initial bladder tumors, but also for concomitantly sampled urine sediment. To our knowledge, the present study is the first to evaluate the usefulness of *TP53*/*FGFR3* phenotype in both bladder tumor and urine sediment. A positive point was the feasibility of mRNA extraction (*TP53* status) and DNA (*FGFR3* status) from urine sample. We were able to achieve a 46% sensitivity and 81% specificity, with a positive predictive value of 94% and a negative predictive value of 37%.

In conclusion, the sensitivity of the *TP53*/*FGFR3* phenotype test in urine does not suggest the replacement of urine cytology and bladder endoscopy combination, however, it could be useful as a complementary tool in the diagnosis of bladder tumors. Our study would have benefited from focusing on a specific cancer subset. Nevertheless, these original data confirmed the feasibility of detection of *TP53* and *FGFR3* mutation in urine samples, which may contribute to increasing the performance of urine cytology. An ongoing study is being conducted in order to further evaluate the usefulness of *TP53* and *FGFR3* mutation testing in urine during monitoring of patients with a history of bladder cancer, particularly regarding early detection of bladder tumor recurrence.

Conflicts of Interest

The Authors declare no conflict of interest with regard to this article.

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