Molecular Diagnosis and Prognosis of Bladder Cancer

Towards the implementation of molecular markers in clinical practice

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Molecular Diagnosis and Prognosis of Bladder Cancer

Towards the implementation of molecular markers in clinical practice

Moleculaire diagnose en prognose van blaaskanker

Op weg naar de implementatie van moleculaire markers in de klinische praktijk

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Alle wetenschap begint met verwondering

Plato

Voor mijn moeder

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PART I

General Introduction

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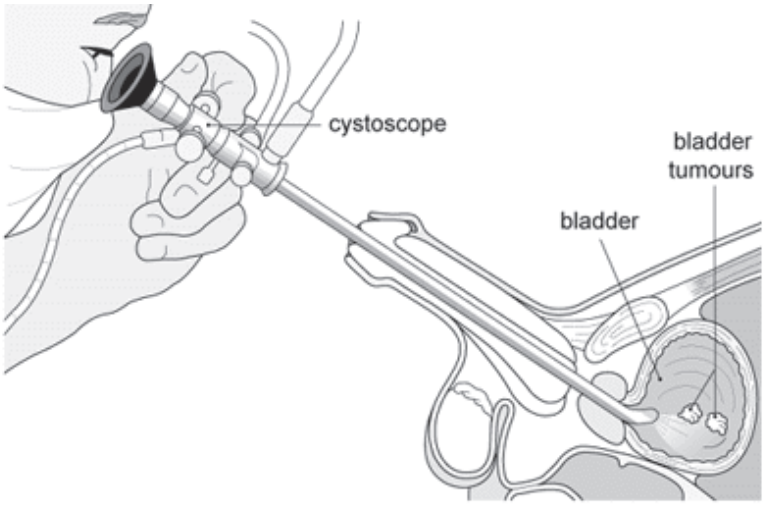
Introduction & Scope of the Thesis

INTRODUCTION

Bladder cancer is the fifth most common malignancy in the Western world after prostate, breast, lung and colorectal cancer. Its incidence directly increases with age reaching a maximum between 60 and 70 years. Bladder cancer is 2.5 times more common in men than in women. The most common bladder carcinogens are aromatic amines. A number of etiological factors are tied to the development of bladder tumors (examples: chronic infections, radiation & chemotherapy, occupational exposure to aromatic amines). However, cigarette smoking is by far the most important risk factor nowadays. More than 90% of bladder cancers are urothelial cell carcinomas (UCC). The other histological types concern squamous cell carcinoma (6-8%) and adenocarcinoma (1-2%). In Egypt and other countries with endemic spread of schistosomiasis, squamous cell carcinoma used to be the most frequent histological subtype. The majority of UCCs is found in the bladder. UCCs of the renal pelvis, ureter and urethra together account for less than 10% of the carcinomas.

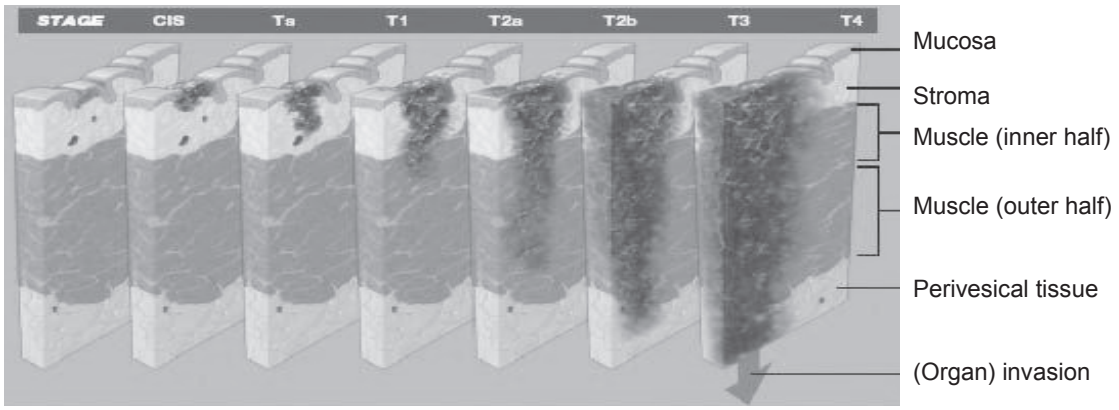
The most important symptom that raises suspicion for a bladder tumor is micro- or macroscopic hematuria. The hematuria is mostly painless and often intermittent which frequently causes a delay in the diagnosis. Behind irritative bladder symptoms such as frequency, urgency or pain, a bladder tumor or flat carcinoma in situ may hide out. The diagnosis of UCC is made endoscopically by urethro-cystoscopy (UCS) and urine cytology (UC). An intravenous urogram and/or CT-scan or ultrasound is needed to visualize the upper urinary tract (renal pelvis and ureters) and the kidneys. UCS allows direct visualization of an exophytic lesion in the bladder. UC particularly aids in the diagnosis of high-grade, flat carcinoma, i.e. carcinoma in situ (CIS). In case of suspicion for UCC in the bladder, a trans-urethral resection (TUR) or biopsy is planned to obtain a histo-pathological diagnosis. Figure 1 shows an example of a UCS before TUR.

Figure 1



Treatment strategies and follow-up mostly rely on the histological Stage (TNM-classification)[1] and Grade (WHO-grading systems)[2,3] of the tumor(s). Figure 2 shows the current TNM-classification for UCC.

Figure 2
TNM 1997 classification for UCC.[1]



CIS: carcinoma in situ. Ta: Non-invasive papillary carcinoma. T1: Tumor invades subepithelial stroma. T2a: Tumor invades superficial muscle. T2b: Tumor invades deep muscle. T3a: Tumor invades perivesical tissue microscopically. T3b: Tumor invades perivesical tissue macroscopically. T4a: Tumor invades prostate, uterus or vagina. T4b: Tumor invades pelvic or abdominal wall.
N0: No regional lymph node metastasis. N1: Metastasis in a single lymph node ≤ 2 cm. N2: Metastasis in a single lymph node >2 cm and ≤ 5 cm, or multiple lymph nodes ≤ 5 cm. N3: Metastasis in a lymph node >5 cm.
M0: No distant metastasis. M1: Distant metastasis.

In approximately 75% of patients, UCC is superficial (i.e. Stages; pTa-pT1, CIS) at first presentation. After TUR, these patients are intensively monitored by cystoscopy because of frequent recurrences and a 10 to 15% chance for progression to invasive, potentially lethal UCC. Patients with intermediate or high risk superficial UCC (multifocal and >pTaG2 disease) usually receive adjuvant intravesical instillations with, in most cases, bacillus Calmette Guérin (BCG) to try to delay recurrence and prevent progression. Patients with persistent high risk (G3) superficial lesions are candidates for a cystectomy. More information on the follow-up of superficial UCC is given in the next paragraph of this chapter. Cystectomy, radiation- or systemic chemotherapy are the preferred treatment options in case of invasive (i.e. ≥pT2) disease depending on presence of metastasis, patients co-morbidity and life expectation. The treatments for invasive UCC have the aim to reduce disease specific mortality. Besides other clinical and pathological parameters, particularly tumor-stage and grade are important for prognosis. Table 1 gives an overall overview of UCC prognosis at first diagnosis. This table also shows that bladder cancer is a heterogeneous disease with an unpredictable clinical course, especially for an individual patient. Nevertheless, patients with superficial UCC have a relatively good prognosis and the greatest concern in these patients is not to reduce mortality but to lower and

postpone recurrent UCCs and to prevent progression to invasive (≥pT2) UCC. On the other hand, many patients diagnosed with invasive UCC and those who progressed to invasive UCC will not survive their disease despite the above mentioned treatment options.

Table 1
Prognosis of Urothelial Cell Carcinoma. Correlation with Tumor-Stage and Grade.

Tumor	Stage/Grade	Recurrence	Progression/ Metastasis	5 years Survival
Superficial	pTa-pT1 G1-2 N0 M0	40-60%	0-5%	90%
Superficial - high risk	pTa-pT1 G3 CIS N0 M0	> 70%	≥ 30%	60-75%
Invasive (after Cystectomy)	pT2-3 N0 M0	-	20-55%	35-65%
Locally Advanced / Metastasized	pT3-4 N1 M1	-	> 60%	< 20%

SCOPE OF THE THESIS:

The general scope of this study will be to evaluate molecular markers for diagnosis in urine and prognosis of UCC in various clinical settings.

The follow-up of superficial UCC requires frequent cystoscopical examinations. Standard practice after TUR of superficial UCC is to follow patients by UCS and UC every 3-4 months for 2 years and every 6-12 months thereafter. The recurrence rate of superficial bladder cancer is up to 75% and progression to invasive disease occurs in around 10-15% of patients, even after long tumor-free periods. This approach is invasive, uncomfortable and costly. Even for flexible UCS, the risk to develop a urinary tract infection is considerable. Urinary cytology is only of limited use because sensitivity is poor, especially for low-grade UCC. For these three reasons, many new urine-based tests for UCC have been developed. Among them, BTAstat, BTAtrak, NMP22, FDP, ImmunoCyt and FISH have been approved by the FDA as adjuncts to cystoscopy. Many other markers are under investigation at this time. The literature on the current urine-based markers for UCC patients who are under surveillance will be reviewed in chapter 2. An important part of the thesis is devoted to the validation of urinary microsatellite analysis, a PCR-based DNA-test, in different clinical settings (chapters 3-5).

Clinical and pathological variables for prediction of patient's prognosis have been studied extensively in various types of cancer, including UCC. The variability in outcome of pathological assessment is a recognized problem, but conventional pathological parameters like grade and stage continue to be one of the best predictors of prognosis. Research efforts of the last decades resulted in a considerable list of molecular investigations for a more accurate assessment of

UCC prognosis. Oncogenes, tumor suppressor genes & associated cell cycle proteins, proliferation antigens, growth factors, cell adhesion molecules and chromosomal alterations involved in bladder carcinogenesis have been identified. Many of these molecular markers hold considerable promise to assess aggressive UCC behavior. Nevertheless, the value of molecular markers over conventional clinico-pathological indices is still being questioned and their clinical use is very limited at this time. The presence of *Fibroblast Growth Factor Receptor 3 (FGFR3)* mutations in a high percentage of UCC was discovered very recently.[4] In part III (chapters 6-12) of this thesis, the frequency, specificity and prognostic value of these mutations in UCC will be studied. The unexpected association of these mutations with UCC of favorable prognosis will be described in this thesis. Furthermore, the possible implications of *FGFR3* mutations for future clinical management, their relation to other molecular- and conventional UCC parameters and the place of these mutations in molecular pathology and disease pathogenesis are some of the items that will be discussed in the chapters of part III.

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Part II

Diagnosis in Urine with Microsatellite Analysis

2

Urine Markers for Bladder Cancer Surveillance

A Systematic Review

Bas W.G. van Rhijn, Henk G. van der Poel, Theo H. van der Kwast

Eur Urol 2005;In press

ABSTRACT

The follow-up of patients with urothelial cell carcinoma (UCC) of the bladder is done by cystoscopy and, in most cases, cytology. The last decade, many urine-based tests for UCC have been developed and tested in different populations. For the urological practice, considering the amount of follow-up cystoscopies, especially urine markers for recurrent disease would be useful. Therefore, we reviewed the literature on these markers for recurrent UCC and compared our findings with recent review-articles. We performed a PubMed search. In case of primary and recurrent disease, the study was included if the patients under surveillance were reported separately. Patients with no evidence of disease at surveillance cystoscopy were considered to determine specificity. A marker was included if at least 2 studies from 2 different institutions/authors were available. The literature review yielded 64 articles. We found 18 markers (BTAstat, BTAtrak, NMP22, FDP, ImmunoCyt, Cytometry, Quanticyt, Hb-dipstick, LewisX, FISH, Telomerase, Microsatellite, CYFRA21-1, UBC, Cytokeratin20, BTA, TPS, Cytology) that met our criteria. BTAstat, NMP22, ImmunoCyt and cytology were evaluated in more than 750 patients. Telomerase, Cytokeratin20 and Hb-dipstick were tested in less than 250 patients. The highest median sensitivities were reported for CYFRA21-1 (85%), Cytokeratin20 (85%) and Microsatellite analysis (82%). The highest specificities were reported for Cytology (94%), BTA (92%) and Microsatellite analysis (89%). In comparison with recent reviews, median sensitivity was ≥5% lower for the surveillance group in 13/18 urine-based tests while specificity remained relatively constant between different patient groups. To our knowledge, this is the first review that assesses sensitivity and specificity of urine markers solely for UCC surveillance. In our view, Microsatellite analysis, Immunocyt, NMP22, CYFRA21-1, LewisX and FISH are the most promising markers for surveillance at this time. Nevertheless, clinical evidence is insufficient to warrant the substitution of the cystoscopic follow-up scheme by any of the currently available urine marker tests. Future studies may test some of the most sensitive and specific assays to reduce the cystoscopy frequency. However, our results show that initiators of these studies should anticipate a lower sensitivity than reported in the current literature.

INTRODUCTION

Around 70% of the patients with urothelial cell carcinoma (UCC) of the bladder initially present with superficial (pTa, pT1 or pTis) disease. These UCC have a high chance of recurrence (60-85%) but more than 80% remains confined to the (sub)mucosa and, therefore, these patients do not influence UCC-related survival.[1] Nevertheless, extensive and long-term follow-up is needed to prevent progression to invasive, potentially lethal UCC. The current standard of care consists of urethro-cystoscopy (UCS), the gold standard, and urine cytology every 3-4 months for the first two years and at a longer interval in subsequent years. This approach is costly, invasive and uncomfortable. Even for flexible UCS,

the risk to develop a urinary tract infection is around 10%.[2] The current follow-up schemes with more than 500.000 UCS per year in the USA for follow-up alone, largely contribute to the fact that total Medicare payments per patient are the highest for UCC compared to other malignancies.[3,4] In addition, especially for the low-grade lesions, urine cytology is of limited value because of operator dependency and a low sensitivity.[5] For these reasons, many new urine-based tests for UCC have been developed. Among them, BTAstat, BTAtrak, NMP22, FDP, ImmunoCyt and FISH (UroVysion) have been approved by the FDA.[6,7]

Initial studies with new markers are mostly promising, but successive reports often fail to show comparable results. Patient selection seems the most likely explanation for the discrepancies between the studies on urine markers. For the urological practice, in terms of cost-reduction and convenience of our patients, particularly markers to detect recurrent disease would be useful. In order to reduce the number of UCS needed for follow-up, the specificity of a urine-based test is important. However, high specificity may be at the cost of sensitivity, conventional cytology being a good example of this.[6] As outlined earlier, positive (percentage in whom the test is positive and the disease is present) and negative (percentage in whom the test is negative and the disease is absent) predictive values are less useful for comparison of two populations with different UCC incidences because they vary by their definition.[6,7] Conversely, sensitivity and specificity stay constant between populations with different numbers of tumor and non-tumor cases and they are more commonly used in studies.[6,7]

Our systematic review of the literature on the performance (sensitivity and specificity) of urine markers was confined to publications on recurrent UCC and we compared our findings with data on the same tests that were reported in reviews without this selection criterion.

METHODS

We performed an online PubMed search up to 2004 to obtain references for the various urine tests. The terms used for the PubMed search included urine, the name of the individual urine-based test, bladder, cancer, urothelial / transitional (cell) carcinoma, tumor marker and recurrence. The material and methods section of each article was screened in order to be sure that the patients were under surveillance with UCS. In case of a mixed study population composed of both primary and recurrent UCC, we only included the particular study if the results of the patients under surveillance were reported separately. Only patients with no evidence of disease at surveillance cystoscopy or pathological evaluation were considered as controls to determine specificity. We did not select on FDA-approval, single or multi-center trials, number of patients involved in the study, tumor grade, urine or bladder wash cytology and/or comparison of more tests in the same study population. A urine marker was included in our review if a minimum of two studies from two different

institutions/authors were available. A comparison was made with recent review-articles to investigate whether our selection criterion (recurrent disease) influenced the performance of the various urine markers.

The usefulness of the urine-based tests for UCC was assessed with sensitivity and specificity. Sensitivity of a test was defined as the percentage of patients with UCC (as defined by the authors) for whom the test is positive (tested positive / patients with recurrent UCC). Specificity was defined as the percentage of patients with a negative cystoscopy in whom the test is also negative (tested negative / no evidence of disease at follow-up).

RESULTS

The literature review on recurrent UCC yielded 64 publications in which we found 18 markers (conventional cytology included) that met our strict criteria. We have listed the studies per marker in the appendix section. Other promising markers (examples: BCLA-4, Survivin, HA-HAase, DD23 and BTF) that were not included in our analysis may also prove to be of value for patients under surveillance and require further study by other groups. Table 1 shows the median sensitivity and specificity as well as the number of studies, institutions and patients.

Table 1

The median sensitivity and specificity, the number of studies, institutions and patients are shown. The data applies to patients under surveillance for UCC. na = not analyzed. See appendix for references.

Marker	studies/ institute (n/n)	no. pts.	median Sensitivity	range (min-max)	no. pts.	median Specificity	range (min-max)
BTAstat	17/14	1377	58	29-74	2084	73	56-86
BTAtrak	4/4	360	71	60-83	195	66	60-79
NMP22	15/12	838	71	47-100	1203	73	55-98
FDP	4/4	168	54	47-68	173	61	25-80
ImmunoCyt	6/5	276	67	52-100	683	75	62-82
Cytometry	5/5	364	60	45-85	89	82	50-92
Quanticyt	5/2	129	58	45-65	227	76	68-87
Hb-dipstick	2/2	117	40	37-41	113	87	87
LewisX	3/2	95	75	68-79	215	85	67-86
FISH	4/4	165	79	70-86	147	70	66-93
Telomerase	3/3	146	39	29-66	-	na	na
Microsatellite	6/4	108	82	75-92	153	89	79-100
CYFRA21-1	3/2	156	85	75-88	323	82	73-95
UBC	5/4	267	60	21-80	480	87	72-95
Cytokeratin20	2/2	117	85	79-87	61	76	76
BTA	5/5	436	48	32-58	216	92	91-92
TPS	3/2	179	65	50-80	246	83	63-95
Cytology	26/20	2213	35	13-75	3322	94	85-100

The number of institutions/authors varied from 2 to 14. BTAstat (N=3461), NMP22 (N=2041), ImmunoCyt (N=959) and conventional cytology (N=5535) were evaluated in more than 750 patients in all studies combined. Telomerase (N=146), Cytokeratin20 (N=178) and Hb-dipstick (N=230) were tested in less than 250 patients.

All the 17 urine markers had a higher sensitivity for recurrent UCC than conventional cytology. The highest sensitivities were reported for CYFRA21-1 (85%), Cytokeratin20 (85%) and Microsatellite analysis (82%). Of the FDA approved tests, FISH (79%), BTAtrak (71%) and NMP22 (71%) had the highest sensitivity. The highest specificities were reported for conventional cytology (94%), BTA (92%) and Microsatellite analysis (89%). ImmunoCyt (75%) had the highest specificity of the FDA approved tests. The specificity (patients in follow-up with no evidence of disease) was not investigated in every study. Specificity was not determined in 4 cytology, 1 BTAtrak, 1 Quanticyt, 2 FDP, 3 Telomerase, 1 Cytokeratin20, 2 Cytometry, 1 Hb-dipstick, 1 NMP22, 1 FISH, 1 BTAstat and 1 BTA studies, respectively. Unfortunately, the rapid point of care tests (BTAstat, FDP, Hb-dipstick and UBC) were associated with a lower sensitivity and specificity (Table 1).

In order to see if our selection criterion (recurrent disease) influenced sensitivity and specificity, we selected several recent reviews.[6-14] The sensitivities and specificities reported in the reviews are summarized in Table 2.

Table 2

Sensitivity and specificity of the data reported in recent reviews are summarized.[6-14] A ≥5% lower median sensitivity or specificity for recurrent disease (Table 1) is marked with a "yes" at the 5% difference column.

Marker	median Sensitivity	range (min-max)	5% difference	median Specificity	range (min-max)	5% difference
BTAstat	70	24-89	yes	75	52-93	-
BTAtrak	69	57-79	-	65	48-95	-
NMP22	73	47-100	-	80	56-95	yes
FDP	61	52-81	yes	79	75-96	yes
ImmunoCyt	83	50-100	yes	80	69-90	yes
Cytometry	60	45-83	-	80	36-87	-
Quanticyt	59	45-69	-	79	70-93	-
Hb-dipstick	52	41-95	yes	82	68-93	-
LewisX	83	80-89	yes	85	80-86	-
FISH	84	73-92	yes	95	92-100	yes
Telomerase	75	7-100	yes	86	24-93	na
Microsatellite	91	83-95	yes	94	89-100	yes
CYFRA21-1	94	74-99	yes	86	67-100	-
UBC	78	66-87	yes	91	80-97	-
Cytokeratin20	91	82-96	yes	84	67-97	yes
BTA	50	28-80	-	86	66-95	-
TPS	72	64-88	yes	78	55-95	-
Cytology	48	31-100	yes	94	62-100	-

In addition, studies with a ≥5% lower median sensitivity or specificity for recurrent disease compared to the numbers extracted from the reviews are also indicated in Table 2. Median sensitivity was ≥5% lower for the surveillance group in 13/18 cytological tests. Telomerase (-36%), UBC (-18%), Immunocyt (-16%), Cytology (-13%), BTAstat (-12%), Hb-dipstick (-12%) and LewisX (-12%) showed a decrease in sensitivity of more than 10%. A higher median sensitivity for the surveillance group was only found for BTAtrak (+2%). Median specificity was ≥5% lower for the surveillance group in 6/17 tests. FISH (-25%) and FDP (-18%) showed a decrease in specificity of more than 10%. Higher specificities were reported for BTA (+6%), Hb-dipstick (+5%), TPS (+5%), Cytometry (+2%) and BTAtrak (+1%). In general, the sensitivity of urine markers is lower for patients who are under surveillance for UCC while their specificity is not much influenced by patient selection.

We also performed a sub-analysis on the sensitivity of the urine markers per pathological grade (WHO 1973, G1-3). Data on the identical set of urine markers were derived from the same articles as listed in Table 1. Table 3 shows the results of this sub-analysis.

Table 3

The median sensitivity per grade (G1-3) and specificity of the urine markers for patients under surveillance are shown. The reference numbers correspond to the ones listed in the appendix section. na = not analyzed.

Marker (reference number)	no.pts. / median Sensitivity			no.pts. / median Specificity
	G 1	G 2	G 3	
BTAstat (1,7,10,11,13,14,16)	228 / 45	206 / 60	208 / 75	972 / 79
BTAtrak (1,2,4)	60 / 55	61 / 59	101 / 74	195 / 66
NMP22 (1,14,15)	56 / 41	77 / 53	81 / 80	235 / 59
FDP (3)	13 / 62	36 / 64	22 / 86	113 / 80
ImmunoCyt (5)	23 / 78	10 / 90	18 / 100	83 / 62
Cytometry (4)	18 / 11	54 / 41	38 / 66	52 / 87
Quanticyt (5)	-	11 / 64	5 / 80	56 / 68
Hb-dipstick (1)	13 / 15	36 / 39	22 / 73	113 / 87
FISH (1,3)	25 / 56	9 / 78	20 / 95	130 / 70
Microsatellite (1,2,3,4,5)	27 / 67	21 / 86	30 / 93	138 / 88
UBC (3)	29 / 38	29 / 41	16 / 69	79 / 72
Cytokeratin20 (2)	14 / 71	35 / 80	35 / 100	na
BTA (2,4)	31 / 16	43 / 47	50 / 52	91 / 91
TPS (3)	29 / 32	35 / 54	15 / 74	72 / 63
Cytology (1,5,7,9,13,14,20,23,24,25)	239 / 17	274 / 34	201 / 58	861 / 95

The sensitivity per grade was not separately mentioned for patients under surveillance in the studies for LewisX, Telomerase and CYFRA21-1. The specificity was not reported in one study on BTAstat (10), Cytokeratin20 (2), BTA (2) and in two studies on Cytology (9,24). The numbers between the parentheses refer to the appendix section. Only 7 of the 18 markers were tested per grade in more than one study with Cytology tested in 10, BTAstat in 7 and Microsatellite

analysis in 5 studies, respectively. The sensitivity increased for every marker with a higher grade. Of the markers that were determined in more than one study, Microsatellite analysis and FISH had the highest sensitivities per grade for patients under surveillance and the specificity remained high for Microsatellite analysis (88%). In two recent reviews the sensitivity per grade was also determined.[8,10] In general, the sensitivity per grade of the urine markers is lower for the patients who are under surveillance for UCC as compared to the figures given by the two reviews on unselected patients.

DISCUSSION

Driven by the low sensitivity of conventional cytology, many research efforts to develop urine markers have been undertaken. These markers generally have a higher sensitivity but a lower specificity than conventional cytology.[8,9] Reviews also showed an increasing sensitivity for urine markers and conventional cytology with the increase of tumor grade.[8,10] After our selection of data on detection of recurrent UCC, we found a ≥5% lower sensitivity for most urine markers when we compared the same markers with the sensitivities reported in recent review articles.[6-14] The reason for this lower sensitivity for recurrent UCC may be the overrepresentation of a small, pTa and low-grade lesions among these recurrent tumors hampering their detection in urine.[15,16] Nevertheless, it is also possible that a given marker has been evaluated by the literature in more recurrent cases than another marker. This may influence the extent of the difference in sensitivity.

The various urine tests have been extensively discussed in the literature.[6-14] Therefore, we will give a short overview per test and we will discuss some advantages and disadvantages. In addition, we will compare the sensitivity and specificity of the investigated markers for UCC surveillance, rather than discussing every marker in detail. The references as well as the manufacturers of each test appear in the appendix section. In the text, the reference numbers appear between parentheses. Cytology is not discussed separately as it was mostly used as reference for a particular assay under investigation.

BTA and NMP22 assays. Three BTA tests (BTA, BTAstat and BTAtrak) are available. BTAstat is a qualitative assay that can be performed in a few minutes, even before cystoscopy, and BTAtrak is a quantitative two-step ELISA assay. They both measure the human complement factor H related protein in urine whereas the original BTA test is a latex agglutination assay that detects basement membrane degradation complexes. NMP22 is a nuclear protein that is responsible for the chromatid regulation and cell separation during replication. This is a quantitative ELISA assay that requires urine stabilization. In our review, the sensitivity and specificity of the original BTA test were comparable to cytology. Compared to the other urine markers, the BTAstat and the NMP22 tests have been extensively studied. Although the sensitivity of BTAstat was slightly higher than that of cytology, specificity was much lower. In addition, benign

inflammatory conditions may give false positive results for BTAstat and NMP22 in over 25% of cases (5,6). The BTAtrak and the NMP22 assays were more sensitive than BTAstat. However, different cutoff values in the reported studies, many false negative cases for G1-2 and median specificities of 66% for BTAtrak and 73% for NMP22 will impede their applicability in the reduction of the number of follow-up cystoscopies.

FDP and Hb-dipstick. As bladder tumors tend to bleed, hemoglobinuria may occur. Moreover, coagulation with the production and degradation of fibrin will occur and, consequently, fibrin degradation products may be found in the urine. Both tests are rapid point of care tests. However, they do not seem to have additional value over cytology for recurrent disease.

Cytometry and Quanticyt. DNA ploidy abnormalities assessed by either flow or image cytometry are important indicators of malignant disease. Flow cytometry only added little information to that obtained by cytology (2,4) and image analysis was also not more sensitive in direct comparison with cytology in two studies (3,5). The Quanticyt system combines ploidy and nuclear morphometry. Though this automated system performed as good as 4 expert cytologists (3), no proof exists that it may reduce the frequency of cystoscopy. The major drawback of cytometry and Quanticyt is the need for a bladder wash sample instead of voided urine.

Immunocytology. The ImmunoCyt test and immunostaining of the LewisX antigen are two examples of immunocytology. ImmunoCyt uses three fluorescent monoclonal antibodies: 19A211, M344 and LDQ10. Initial data were promising (1,3), but successive reports failed to confirm the high sensitivity of ImmunoCyt (2,6). A large French multicenter study concluded that the ImmunoCyt test improved the diagnostic accuracy of cytology in daily practice (4). However, a high inter-observer variability (2) and a 16% lower sensitivity for recurrent UCC may hamper the clinical utility of the assay. Unfortunately, the sensitivity per grade was only determined by one study (5). LewisX antigen had a higher sensitivity (75%) and a good specificity (85%) compared to cytology. However, only 3 studies of two institutions were available. Expression of LewisX antigen by benign umbrella cells in the urine may interfere with the results of the assay. Additional studies by other groups are needed for both tests to determine whether these tests indeed have sufficient sensitivity and specificity to be used in a study protocol to reduce the cystoscopy frequency.

Cytokeratin (CK) based assays. We reviewed 4 CK assays. CKs are intermediate filament proteins specific for epithelial cells. A given epithelium can be characterized by a chain-specific CK expression pattern. In general, overexpression of a particular chain-specific CK is associated with UCC. UBC is available as an ELISA assay (1-3,5) that takes two hours to perform or as an on bench test that gives an immediate result (4). Both tests detect traces of CK8 and CK18 in urine. TPS also detects CK18 and requires incubation with a second monoclonal antibody (M3 fragment of CK18). If we compare our sensitivity data

with other reviews we observed a decrease in sensitivity of 18% for UBC and 7% for TPS. Furthermore, the studies by Sanchez et al. (1,2) and Boman et al. (3) showed comparable sensitivity and specificity for UBC, TPS and NMP22. CYFRA21-1 is an ELISA that detects fragments of CK19 with the help of two monoclonal antibodies (BM19.21 and KS19.1). Three studies from two institutes reported high sensitivity and specificity for this test. Urinary stones, infection and previous intravesical treatment with BCG caused many false positive results (3). In addition, Sanchez et al. (1,2) reported similar results for NMP22 and CYFRA21-1. CK20 is selectively expressed in bladder and gastrointestinal epithelia. Only two studies were available. CK20 expression was determined by RT-PCR techniques (2) or as an immunocytological assay (1). Initial results are promising but CK20 was not compared to urine markers other than cytology. In conclusion, CK based assays, particularly CYFRA21-1, are promising. However, the number of studies is relatively low, cutoff values have not yet been defined properly and the additional value over NMP22 is not obvious. Clearly, further study by more institutions will be required before these tests may be considered eligible for studies to reduce the number of follow-up cystoscopies.

Genetic Alterations. Genetic instability is an important feature of malignancy. We reviewed 3 genetic assays. Microsatellites are highly polymorphic tiny DNA fragments that are frequently found throughout the genome. UCC is characterized by frequent loss of heterozygosity (LOH) at the chromosomal locations: 4p, 8p, 9pq, 11p and 17p. Microsatellite analysis combines 15 to 20 markers from a region with a high percentage of LOH. This method proved very sensitive for low and high grade lesions with sensitivities of 67%, 86% and 93% for recurrent G1, G2 and G3 lesions, respectively, and a specificity of 88%. Moreover, Microsatellite analysis could predict a recurrence before cystoscopical evidence in all 3 studies with extended follow-up (1,2,4). Although Microsatellite analysis has a very high potential, automation of the assay, multi center studies and excluding patients with persistent leukocyturia are essential if this analysis is to become clinically important. Fluorescence in situ hybridization (FISH) with 4 multitarget probes to the centromeres of the chromosomes 3, 7 and 17 and to the 9p21 band form the FISH UroVysion assay. Polysomy or the presence of 9p21 loss indicates cancer positivity. The assay was more sensitive than BTAstat and capable of predicting a future recurrence (1). Nevertheless, a specificity of 70% for patients under surveillance is not acceptable to reduce the number cystoscopies. However, as FISH was capable of predicting an early recurrence in patients with a negative UCS, specificity may be underestimated in the context of surveillance. Further study is clearly indicated for this test. Telomeres consist of identical short repetitive DNA sequences at chromosomal ends. Telomere length decreases with age, unless telomerase is present to prevent this process. Renewed expression of telomerase occurs in most cancers and is thought to immortalize cancer cells.

However, for the detection of recurrent UCC, the assay lacked sufficient sensitivity.

CONCLUSIONS

Non-invasive tests for UCC have many potential applications including the diagnosis of recurrent UCC. With the help of a urine marker, the bother for the patients and costs of follow up may be decreased. If we pursue a 100% sensitivity and specificity for a urine test, the discussion can be short because such a marker does not exist and UCS, the gold standard, is also not 100% sensitive and specific. However, if we consider the follow up of superficial UCC, we may discern patients with low grade (G1-2) superficial lesions from the patients with high grade (G3/CIS) disease. High grade tumors should be detected early and the percentage of tumors missed should be as low as possible. Therefore, the optimal approach for these patients will continue to include both frequent UCS and cytology as an adjunct to detect invisible disease. If this follow up is to include a urine marker other than cytology, is a question difficult to answer. Specificity is more important than sensitivity in this subset of UCC patients as the urine marker is used as an adjunct to UCS. On the other hand, for patients with low grade UCC, it might be acceptable to postpone the diagnosis of recurrent low grade tumors. Although a randomized multi-center study with a lower follow up frequency has not been done, low progression rates[1,17] and the safety of the marker lesion model to evaluate the anti-tumor activity of intravesical drugs[18], support this idea. Moreover, Schrag et al., who analyzed the adherence to surveillance among patients with superficial UCC in a 3 year follow-up period, found that only 40% of patients had undergone all cystoscopical examinations as recommended by the clinical guidelines.[19] If we consider the follow up of low grade UCC, the first target for a urine marker is the reduction of the number of UCS needed for follow up by alternation of UCS with a urine test. Using such an approach, sensitivity and specificity of a urine marker are both important because a low sensitivity will eventually miss too many tumors and a low specificity may lead to unnecessary invasive procedures to rule out the presence of UCC in the upper urinary tract. As a randomized study with a urine marker has not been done in this patient group, it is very difficult to choose a particular urine marker or to comment on which sensitivity and specificity levels are required for this purpose. As discussed in this review, all the urine markers have their advantages and disadvantages. For example, some markers have been investigated in many patients and/or give instant test results (BTAstat, FDP, Hb-dipstick and UBC) but are associated with lower sensitivity and specificity whereas other markers have a higher sensitivity and specificity but require more extensive studies for validation. This does not imply that urine markers are completely useless on an individual basis at this time. In patients with low grade superficial UCC, these tests may help in planning a follow-up UCS. However, one should realize that this approach is not supported by sufficient clinical

evidence at this time. In our view, Microsatellite analysis, CYFRA21-1 and LewisX are the most promising non-FDA approved urine markers while ImmunoCyt, NMP22 and FISH are the best FDA approved ones for surveillance. The choice for these 6 markers was not only based on sensitivity and specificity but also on the other items mentioned in the discussion-section. Future studies for urine markers should use these assays in combination with a lower UCS frequency. Only in this way, the question, which assay - if any - is the one of choice can be answered. Nevertheless, considering the study of Schrag et al.[19], already many urologists and patients seem to have chosen for a lower UCS frequency. A randomized trial will prove if this is feasible and whether a urine marker is helpful in the follow up of superficial UCC.

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APPENDIX

BTAstat, Bladder Tumor Antigen (Polymedco Inc., New York, NY, USA)

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BTAtrak, Bladder Tumor Antigen (Polymedco Inc., New York, NY, USA)

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NMP22, Nuclear Matrix Protein 22 (Matritech Inc., Newton, Massachusetts, USA)

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FDP, Fibrin/Fibrinogen Degredation Products (AuraTek&Accu-Dx, Intracel Corp., USA)

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FISH, Fluorescence In Situ Hybridization (UroVysion by Vysis, Downers Grove, Illinois, USA)

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Telomerase

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Microsatellite

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Microsatellite Analysis — DNA-Test in Urine Competes with Cystoscopy in Follow-Up of Superficial Bladder Carcinoma

A Phase II Trial

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ABSTRACT

It has been shown that microsatellite analysis (MA) is able to detect bladder cancer in urine. Relatively small groups of patients with often high stage and grade disease were investigated so far. However, more than 85% of cystoscopies are performed for follow-up of superficial bladder cancer. We evaluated this DNA-based method in a group of consecutive patients in follow-up after trans-urethral resection of superficial disease. Matched blood and urine samples from 109 patients were obtained before cystoscopy and subjected to MA. The BTAstat test and cytology were used for comparison. Sixteen patients were excluded: the DNA was of insufficient quality in 7 patients and leukocyte abundance rendered the result of MA unreliable in 9 patients. In the remaining 93 patients, MA detected 18 of the 24 recurrent tumors. The 6 undetected tumors were small pTaG1-lesions for which immediate surgery was not necessary. On the other hand, 5/9 patients with a positive MA and a negative cystoscopy had a tumor-recurrence within 6 months after urine collection. In contrast, a recurrence occurred in only 7/60 patients who were negative in both MA and cystoscopy (P=0.006). The MA (74%) appeared more sensitive than the BTAstat test (56%) or urine cytology (22%). MA is a DNA-test in urine that reliably signals the presence of recurrent bladder cancer, sometimes even before cystoscopical evidence of the disease. This non-invasive diagnostic tool has the potential to replace cystoscopy in many cases. Our results warrant the need for randomized trials.

INTRODUCTION

Bladder cancer is the fifth most common neoplasm in the western world with approximately 54.300 new cases in the USA in 2001.[1] The patients require extensive follow-up after initial treatment. Invasive bladder cancer (i.e. ≥pT2) is usually treated by cystectomy in case of non-metastatic disease. However, in the majority of patients, bladder cancer is superficial (i.e. pTa, pT1, Tis) at first presentation. Standard practice after trans-urethral resection (TUR) of superficial bladder cancer is to follow patients by cystoscopy every 3-4 months for 2 years and every 6-12 months thereafter. The recurrence rate of superficial bladder cancer is up to 75% and progression to invasive disease occurs in around 15-25% of patients, even after a long tumor-free period.[2,3] Urinary cytology is only of limited use since sensitivity is poor, especially for low-grade bladder cancer. To enhance sensitivity, tests in urine, such as bladder tumor antigen (BTA) and nuclear matrix proteins determination (NMP22), have already been approved by the Food and Drug Administration as alternatives for cytology. However, they fail to diagnose recurrent bladder cancer with sufficient accuracy in order to change the current cystoscopy regimens.[4-7]

Microsatellites are highly polymorphic DNA repeats that are frequently found throughout the human genome. Microsatellite markers can detect cancer-associated genetic alterations such as loss of heterozygosity (LOH) and

microsatellite instability (MSI). Bladder cancer is characterized by a wide variety of genetic changes reflected by frequent LOH and MSI.[8-10] Chromosomes 4p, 8p, 9p, 9q, 11p and 17p often display LOH. Particularly the role of chromosome 9 is important for follow up purposes, as it is frequently affected in superficial as well as in invasive bladder cancer.[8,9,11] The group of Sidransky previously showed that bladder-, upper tract urothelial and renal cancer could be detected by microsatellite analysis (MA) of urine.[10,12-15] The same genetic alterations that were found in tumor DNA were also found in the DNA derived from the urinary sediment in these previous studies. These and other studies[16,17] also provide evidence for a monoclonal origin of bladder cancer, which makes the use of microsatellite markers particularly suitable for the detection of bladder cancer in urine.

Encouraging high sensitivities for the detection of recurrent bladder cancer in urine by MA were reported in two independent investigations, limited to relatively small subsets of patients.[14,18] In the daily clinical practice more than 85% of the cystoscopies are performed in order to detect recurrent bladder cancer in patients who were previously diagnosed with superficial bladder cancer. The present study evaluates this novel, non-invasive molecular approach in a group of consecutive patients who are in follow-up after TUR of superficial bladder cancer.

PATIENTS AND METHODS

Patients. We included 109 consecutive patients in follow-up after TUR of superficial bladder cancer after written informed consent was obtained. The mean age was 67.3 ± 11.4 years at time of the study entrance; the male/female ratio was 3.4:1. In addition, eight age-matched patients who underwent cystoscopy for benign urologic diseases were included as controls. All the patients were seen at the outpatient clinic of the University Hospital Rotterdam. The medical-ethical committee of the Erasmus University and the University Hospital Rotterdam approved the study (MEC 168.922/1998/55). The mean number of previous superficial tumors was 2.6 ± 3.0 and varied from 1 to 21 tumors per patient before inclusion. The patients were followed after complete TUR according to the follow-up scheme applied in our hospital: cystoscopy every three to four months for the first 2 years and every six to twelve months thereafter. The grading of cancers was performed according to the WHO classification and the staging according to the 1997 TNM classification guidelines (UICC 1997). The highest tumor-stage in the past was pTa, pT1 and carcinoma in situ (CIS) in 64, 41 and 4 patients, respectively. The highest tumor-grade in the past was G1, G2 and G3 in 27, 45 and 37 patients, respectively. We also obtained 6 months clinical follow-up after MA.

Sample collection and DNA isolation. Before cystoscopy, freshly voided urine samples (15-50 ml) and heparinized venous blood (7 ml), as a source of reference DNA, were collected from each patient and stored at +4°C. The urine

samples were spun at 3,000 rpm for 6 min within 5 hours after voiding. The pellet was rinsed with 10 ml phosphate-buffered saline (PBS) and spun again at 3,000 rpm for 6 min. The pellet was then resuspended in 800 μl PBS, brought to a 1.5 ml eppendorf-tube and spun down at 10,000 rpm for 2 min. The cells were stored at -80°C until DNA isolation. DNA from the urine-pellets was extracted using the QIAamp® Viral RNA kit (Qiagen GmbH, Hilden, Germany) according to the enclosed protocol, isolating both DNA and RNA at the same time. DNA from venous blood was isolated using the one-step DNAzol® BD Reagent (Life Technologies, Inc. [Gibco BRL], Grand Island, NY). This DNA was stored at -30°C until use.

Microsatellite analysis. Microsatellite markers were selected on their performance on control DNA, informativity, frequency of LOH and MSI in bladder cancer on different chromosomal arms[8,9,12,14,15,18] and a limited number of annealing temperatures for high throughput purposes. The 19 markers and their characteristics are depicted in table 1. Polymerase chain reaction (PCR) was performed as indicated by the manufacturer of Taq-polymerase (Promega, Madison, WI). PCR-products were separated on denaturing 6% polyacrylamide-urea gels. Autoradiography was performed overnight at -80°C with Fuji Super RX film (Fuji Photo Film Co., Ltd., Tokyo, Japan) and followed by a quantitative analysis to determine the allelic-imbalance (AI) using the PhosphorImager system and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). LOH was scored in informative cases if a reduction of 30% in the ratio of the signals from urine alleles was calculated in comparison with corresponding reference (blood) alleles. MSI was scored if a deletion or expansion of a repeat unit was found as a new band. Every microsatellite alteration (LOH and/or MSI) was confirmed by a second PCR and the scoring was done independently (BvR, IL) without the knowledge of clinical or pathological data.

BTAstat and urine cytology. The BTAstat test was done if the urologist applied for the test. The BTAstat test (Bard Diagnostic Sciences, Inc., Redmond, WA) was performed by placing five drops of urine into the sample well of a disposable test device. The result was read 5 minutes after placement of the urine according to the enclosed protocol. The urine for cytology was concentrated by centrifugation. The cells were stained by the standard Papanicolaou staining procedure. The final diagnosis from the cytopathology department of our hospital was used. The 3 tests were carried out on the same urine specimen.

Statistical analysis. The statistical package for social sciences 8.0 (SPSS Inc., Chicago, IL) computer software was used for the data documentation and analysis. Sensitivity (positive urine-tests / recurrent bladder cancers) and specificity (negative urine-tests / no evidence of recurrent bladder cancer assessed by cystoscopy or biopsy) were calculated. The two sided Fisher's exact test was used to determine if a urine-test could predict tumor

recurrence within 6 months in the patients who were free of disease at urine sample collection.

Table 1

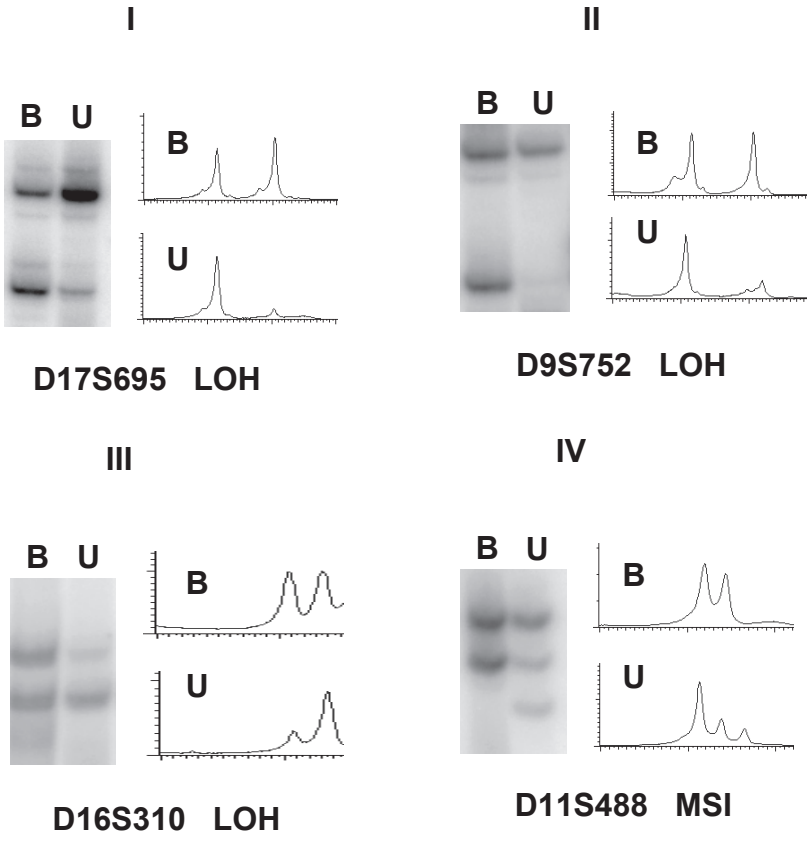
The 19 microsatellite markers used for DNA-analysis in urine. Overview of chromosomal location, type of repeat, different annealing temperatures (Temp.) and primer sequences. Data retrieved from the Genome Database; http//www.gdb.org/gdb-bin/

Marker	Locus	Type of repeat	Annealing Temp °C	Primer sequence 5' – 3'
ACTBP2	5p	tetra	55	AATCTGGGCGACAAGAGTGA ACATCTCCCCTACCGCTATA
FGA	4q28	tetra	55	CCATAGGTTTTGAACTCACAG CTTCTCAGATCCTCTGACAC
D16S476	16q	tetra	55	TTGCACTCCACTCTGGGCA TTGCCTTGGCTTTCTGTTGG
D18S51	18q21.33	tetra	55	GAGCCATGTTCATGCCACTG CAAACCCGACTACCAGCAAC
D4S243	4pq	(CA)n	55	TCAGTCTCTCTTTCTCCTTGCA TAGGAGCCTGTGGTCCTGTT
D9S162	9p22-9p21	(CA)n	55	GCAATGACCAGTTAAGGTTC AATTCCCACAACAAATCTCC
D9S242	9q32-9q33	tetra	55	ACTCCAGTACAAGACTCTG GTGAGAGTTCCTTCTGGC
D9S252	9q13-9q22	tetra	55	ACAATGAACATCCATATACCC ACCATGATTTGTCAACTCCTA
D11S488	11q24.1-11q25	tetra	55	GCCTAGGCAATAGAGACCC GAATTGTTACTGAGAGTTAG
D9S171	9p	(CA)n	55	AGCTAAGTGAACCTCATCTCTGTCT ACCCTAGCACTGATGGTATAGTCT
D16S310	16q	tetra	60	GGGCAAGGAGAGACTCT AAAAAAGGACCTGCCTTTATCC
THO	11p	tetra	60	CAGCTGCCCTAGTCAGCAC GCTTCCGAGTGCAGGTCACA
D9S752	9q33-9q34	tetra	62	CAGAGGTTGCAGTGAGCTA GCAAAGTCAGGCCATTATAC
D17S695	17p	tetra	62	CTGGGCAACAAGAGCAAAATTC TTTGTTGTTGTTCATTGACTTCAGTCT
LPL	8p22	tetra	62	ATCTGACCAAGGGATAGTGGGATATA CCTGGGTAACTGAGCGAGACTGTGTC
D9S144	9p	(CA)n	55	AAATATTATAGCAAGTTAATTACTGAA GGATAAATACACTGGAAAAGAGAT
D20S454	20pq	tetra	55	TGAAGTGTAGAGCTTGACA TGCAGTGAGCCATGTTCAT
D17S786	17p	(CA)n	55	TACAGGGATAGGTAGCCGAG GGATTTGGGCTCTTTTGTAA
D17S960	17p	(CA)n	55	TGATGCATATACATGCGTG TAGCGACTCTTCTGGCA

RESULTS

Microsatellite analysis. We tested urine-DNA samples from 109 consecutive patients who were in regular follow-up after resection(s) of superficial bladder cancer and from 8 control patients without bladder cancer. For the MA we used a panel of 19 microsatellite markers comparing DNA isolated from urine and control DNA from blood of the same patient. Several representative microsatellite migration patterns from gel-electrophoresis and the calculation of allelic-imbalance ratios to determine LOH are shown in figure 1. The 8 control patients without bladder cancer showed no microsatellite alteration in their urine DNA. We had to exclude 16 of the 109 consecutive patients because of the following reasons. Firstly, the amount of urine DNA was not enough for reproducible PCR results in 7 patients. All these 7 patients were tumor-free at the time of urine analysis. The design of the study did not allow us to obtain a second urine sample of the same patient. Secondly, in another 9 patients, 20 or more leukocytes were seen per microscopic view (magnification 400x). In these 9 patients, 4 of the 5 tumor-recurrences were undetectable due to contamination by the “normal” leukocyte DNA. Possibilities to circumvent this drawback are addressed in the discussion section.

Figure 1



We discovered at least 1 microsatellite alteration in 27 of the remaining 93 patients (table 2). The mean number of altered microsatellite markers was 3.0 ± 2.6 and varied from 1 to 9 per patient. LOH accounted for 88% of the microsatellite alterations; a MSI was only observed 10 (12%) times in a total of 7 patients and 6 of these 7 patients also showed LOH. Our data indicate that MSI may not be as frequent as initially reported in bladder cancer.[10] Our observations were more in agreement with the study by Bonnal et al.[19] The cystoscopy results in relation to MA and tumor-characteristics at time of urine sample collection of the 93 patients are shown in table 2. The MA detected bladder cancer recurrence in 18/24 (75%) patients at time of urine collection. The 6 tumors that were missed were all small (<0.5 cm) pTaG1 lesions and therefore acute surgical treatment was not necessary. The 18 tumors that were detected by MA were in 15 cases G2 or G3 and consequently in need for surgery on short term. Three of the 24 patients had concomitant CIS and one patient had CIS only. All these 4 patients were also positive in the urinary MA. In the 3 patients with concomitant CIS, it remains unclear whether the microsatellite alteration(s) originate from the CIS or the papillary lesion in the bladder.

All the 69 patients without evidence of disease (negative cystoscopy and T0; table 2) at time of MA underwent at least one cystoscopical examination during 6 months clinical follow-up. We detected 9 microsatellite alterations in these 69 patients resulting in a specificity of 87% (60/69). However, five of the 9 (56%) patients with a positive MA but a negative cystoscopy had a tumor recurrence within 6 months after MA. We were able to obtain DNA from tumor tissue in 4 of 5 cases. In these 4 patients, the MA pattern in tumor matched with the previous urine profile.

Legend of figure 1

I to IV: 4 representative examples of the microsatellite analysis in urine. The migration patterns from gel electrophoresis are shown on the left side. Paired lanes are normal blood DNA (B) and urine DNA (U). The corresponding calculation of the allelic-imbalance ratios is shown on the right side. The Allelic Imbalance (AI) was calculated by division of the ratio in urine DNA (U) and the ratio in blood DNA (B). Loss of Heterozygosity (LOH) was scored if the AI was ≥1.43 or <0.70, depending on loss of the upper or lower allele. Every microsatellite alteration (LOH/MSI) is indicated by an arrowhead in the gel-migration pattern and in the AI-calculation figure. The notation of the microsatellite marker and the appearance of LOH or MSI in this particular marker are indicated below.

The AI in patient I was 8.09 (urineDNA 6.63 / bloodDNA 0.82), indicating LOH of the lower allele. A recurrent lesion was detected at simultaneous cystoscopy and a pTaG3 tumor was removed at subsequent TUR.

The AI in patient II was 7.14, also indicating LOH of the lower allele. In this case a pTaG1 tumor was removed at the same time.

The AI in patient III was 0.38, indicating LOH of the upper allele. In this patient a pT2G3 tumor was removed, but the microsatellite analysis of urine already pointed at the presence of tumor activity 6 months before clinical diagnosis.

A MSI was discovered in patient IV. A pTaG2 tumor was removed at the same time.

The stage and grade distribution of the 5 tumors detected by MA in advance of cystoscopy was pTaG1, pTaG2 (twice), pT1G2 and pT2G3. The detection in urine of the invasive pT2G3 tumor before cystoscopical evidence of tumor activity is of particular interest in this respect. In contrast, a recurrence was found in only 7/60 (12%) patients negative in both MA and cystoscopy. Those 7 recurrences were pTaG1 in three, pTaG2 in three and pTaG3 in one patient. The P-value to determine if MA could predict a tumor recurrence within 6 months after MA in these patients was 0.006 (two-sided Fisher's Exact test). The corrected specificity was 94% (60/64).

Table 2

Cystoscopy, microsatellite analysis and tumor-characteristics of the remaining 93 patients at time of urine sample collection.

	Grade of recurrences			Tumor size		Microsatellite analysis in urine		
	G1	G2	G3	<0.5 cm	≥0.5 cm	No LOH/MSI	LOH/MSI	Total
Negative Cystoscopy	-	-	-	-	-	55	7	62
Positive Cystoscopy								
T0	-	-	-	-	-	5	2	7
pTa	9	5	1	10	5	6	9	15
pT1	0	1	1	0	2	0	2	2
Tis	0	0	1	1	0	0	1	1
≥pT2	0	0	6	1	5	0	6	6
Total	9	6	9	12	12	66	27	93

LOH : loss of heterozygosity, MSI: microsatellite instability, Tis = carcinoma in situ

Comparison with BTAstat and urine cytology. In 72 of the 109 included patients, data on the BTAstat test, conventional cytology and MA were available for a comparison of the 3 tests in urine. Five patients with leukocyte abundance (>20 per microscopic view) were excluded. In these 5 patients, two tumor recurrences (pTaG1 and pT2G3) were discovered. The MA was negative in 5 cases; the BTAstat test was positive in 5 cases. Only the pT2G3 tumor was found by cytology and the other 4 urine samples were negative at cytological examination. Thus, leukocyte abundance interfered with 2 of the 3 tests in urine, resulting in false negative MA and false positive BTAstat tests.

The results of the 67 patients that could be evaluated for the performance of the 3 tests are shown in table 3. The MA (74%) reached the highest sensitivity of the 3 tests in urine. The sensitivities of the BTAstat test and conventional cytology for detection of recurrent bladder cancer were 56% and 22%, respectively (table 3). Moreover, the MA was the only test in urine that could predict the presence of tumor-activity while at the same time, cystoscopy or biopsy showed no evidence of the disease (P=0.009, table 3).

Table 3

Data on the 3 bladder cancer tests in urine for 67 of the 93 patients.
Sensitivity and specificity for detection of recurrent bladder carcinoma at time of urine sample collection are shown as well as prediction of recurrence in patients who were free of disease at time of urine sample collection (n=44).

	At urine sample collection		Recurrence within 6 months after test in urine		
	Sensitivity (%)	Specificity (%)	NED, Urine test positive (%)	NED,Urine test negative (%)	p-value*
Microsatellite analysis	17/23 (74)	36/44 (82)	5/8 (63)	5/36 (14)	0.009**
BTAstat test	13/23 (56)	35/44 (79)	3/9 (33)	7/35 (20)	0.402
Urine cytology	5/23 (22)	42/44 (95)	0/2 (0)	10/42 (24)	1.000

NED = No Evidence of Disease at time of urine-sample collection as assessed by cystoscopy or biopsy
** P-values were calculated for 2x2 tables by Fisher's Exact test (2-sided).*
*** Note that this P-value indicates that the real sensitivity and specificity of microsatellite analysis are higher than the percentages calculated at urine sample collection.*

DISCUSSION

It can be calculated on the basis of incidence numbers and recurrence rate,[1-3] that more than half a million cystoscopies per year for superficial bladder cancer follow-up are performed in the USA alone. A reduction of the number of cystoscopies based on a reliable, non-invasive test in urine would greatly benefit the patients and may also reduce the costs of monitoring.

We evaluated the possible clinical utility of a multi-marker DNA test, called microsatellite analysis (MA), in urine to provide a non-invasive diagnosis of recurrent bladder cancer. The present study is to our knowledge the first phase II trial conducted in a cross-section of a superficial bladder cancer population who undergoes frequent cystoscopy. We reported a sensitivity of 75% (18/24) in our patient group. The previous phase I studies on urinary MA by Steiner et al.[14] and Mourah et al.[18] reported sensitivities of 91% (10/11) and 83% (10/12), respectively. However, those studies included patients with previous invasive as well as superficial bladder cancer. Our somewhat lower sensitivity can be explained by the fact that we solely included patients with superficial bladder cancer. These tumors generally are of lower grade and display a lower number of genetic alterations.[8,9,20] Nevertheless, we were able to diagnose all recurrent tumors that were staged and graded higher than pTaG1. Possible reasons for a lower sensitivity of MA for small pTaG1 lesions may be a scarcity of genetic alterations or the tumors may be just too small to detect a microsatellite alteration in the corresponding urine.[14] However, the tumors missed by MA proved to be relatively innocent, i.e. small pTaG1, tumors that are not in need of immediate surgery. Some studies indeed advocate a lower follow-up frequency for these patients.[21,22]

We reported a specificity of 87% (60/69) in our series. However, five of the 9 patients with a positive MA result without evidence of bladder cancer, appeared to develop an evident bladder tumor recurrence within 6 months. This result, together with the matching MA patterns of urine and the later removed tumor, indicated that sensitivity and specificity of MA are underestimated if cystoscopy is considered the golden standard for tumor detection. The real specificity was 94% (60/64). Our results were in agreement with the specificities mentioned in the previous studies.[14,18] In their study, Steiner et al. also found 2 tumors in advance of cystoscopy and the tumor they could not detect was also a pTaG1 lesion.[14] The considerable value of MA is also evident from the grade 2/3 status of 4 tumors that were detected in advance of cystoscopy. One of these patients had even developed an invasive pT2G3-tumor. Several molecular markers, like TP53 and RB, which indicate bladder tumors with a tendency towards a more invasive tumor behavior, have been described.[16,23] In the future, these markers may be included in the urinary DNA analysis in order to serve as prognostic indicators. An explanation for the detection of tumors by MA that are not visible at cystoscopy is the possibility that flat lesions, like CIS, exfoliate easily, yet remain undetected by the cystoscopist.[24] Furthermore, the frequent occurrence of genetic alterations in normal looking urothelium adjacent to or distant from the histologically confirmed bladder tumor,[11] even in patients diagnosed with low stage and grade papillary tumors,[25] is of interest in this respect. These studies indicate that larger areas in the bladder are affected by genetic alterations than the tumor site(s) alone. In addition, tumors originating from the upper urothelial tract may also cause microsatellite alterations in absence of bladder cancer.[13,15] This item will also be addressed further on in this section.

Cytology and the BTAstat test were used for comparison with MA of urine. The sensitivity and specificity of both tests was too low to justify a less frequent cystoscopy regimen in our patient-population. The low sensitivity of conventional urinary cytology in the follow up of superficial bladder cancer is known and was recently confirmed in a large series of 1672 patients.[26] A reason for a higher sensitivity of MA in comparison with urine cytology may be the earlier mentioned areas of normal looking bladder epithelium with cancer associated genetic alterations.[11,25] Although the BTAstat test is a simple and quick test kit, the literature sustains that the test lacks sufficient sensitivity to detect recurrent bladder cancer.[5-7,27]

Some important issues concerning the MA of urine should be considered in future investigations. Firstly, the design of our study did not allow us to obtain a sufficient quantity of urine in all consecutive patients, probably because they are nervous prior to their cystoscopical examination. Consequently, not enough DNA could be isolated from the urinary sediment for reproducible PCR result in 7 of the 109 patients. Obtaining a larger urine sample one or two days prior to cystoscopy may solve this problem. Since these 7 patients were all tumor-free, it is also possible that insufficient DNA was obtained because normal cells may

have a shorter lifespan in urine and/or shed less easily than cancer cells as hypothesized by Steiner et al.[14] Secondly, leukocytes impose a problem for bladder cancer detection in urine. The present study shows that the abundance of leukocytes interferes with at least 2 of the 3 tests that we performed in urine. This problem has not been mentioned in the previous studies on MA.[10-15,18] The number of patients was probably too small in these studies. The interference of inflammatory conditions with the BTAstat test is mentioned by Sharma et al.[27] A second urine sample will have to be obtained for instance one week later in these patients. In case of persistent leukocyturia, antibiotic therapy might be considered and finally, future studies should investigate if the separation of leukocytes from urothelial cells in the laboratory is feasible.

Another attractive possibility of the urinary MA would be the screening of the upper urothelial tract for the presence of neoplastic lesions. The incidence of upper tract urothelial cancer will probably rise in the near future since the population ages and intravesical treatment, especially bacillus Calmette Guérin (BCG), has proven to be effective to prevent bladder cancer recurrence and progression.[28] In a retrospective study with a median follow-up of 12 years, 25% (78/307) of the patients with initial superficial bladder cancer developed a recurrent tumor in the upper urothelial tract.[29] The upper tract urothelium is currently monitored by an intravenous urogram (IVU) every one to two years. However, an IVU does not allow a direct view of the urothelium and is therefore less sensitive than cystoscopy with regard to cancer detection. This also may explain the high percentage (77%) of invasive tumors among upper urothelial tumors.[29] Two studies already showed that it is possible to detect upper urothelial cancer by MA of voided urine.[13,15] Therefore, patients with a microsatellite alteration in their urine but without cystoscopical evidence of the disease might have a so far undetected lesion in the upper tract urothelium and the MA may identify the patients at a relative early stage of the upper tract disease. Thereby, MA of urine might even prevent cancer-related death. In addition, other malignancies originating from the urogenital tract can also be detected by MA of the urine sediment.[13,18]

We have demonstrated that MA of the urinary sediment is able to compete with cystoscopy in a group of consecutive patients with a history of superficial bladder cancer. In line with our findings, a positive MA justifies a cystoscopic examination and additionally, the data suggest that a cystoscopic examination may be omitted if MA is negative. Before clinical introduction of this molecular diagnostic tool for bladder cancer, the results of this study need to be confirmed in larger cohorts of (randomized) patients. In a future randomized trial, possible pitfalls like insufficient DNA quality, abundance of leukocytes in the urinary sediment and lesions of the upper urothelial tract should be recognized and accounted for in the randomization schemes. Future study should also confirm our suggestion that the frequency of cystoscopy can be reduced considerably based on MA of urine. Nowadays, up to 4 cystoscopies per patient are needed

per year to monitor superficial bladder cancer. The present study is to our knowledge the first phase II trial and it provides a basis for a randomized phase III trial that addresses important issues like cost savings and the anticipated quality of life improvement for these patients. For the USA alone, the cost-savings would amount to 140 million dollars per year if the cystoscopy frequency could be reduced by 50%.[30] Moreover, the molecular analysis may also detect cancers in the upper urinary tract at an earlier stage, which may contribute to an increase in survival for this particular patient group.

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4

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

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ABSTRACT

Fibroblast growth factor receptor 3 (FGFR3) mutations were recently reported 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. 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 & 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. 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 one of these was also detected in the urine. This was 125/357 for the 53 cases without leukocyte contamination. The sensitivity of MA on voided urine was lower for *FGFR3* positive UCC (15/21; 71%) as compared to *FGFR3* wild-type UCC (29/32; 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 14/15 (93%) for the two (molecular and morphological) cytological approaches. 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

Urothelial cell carcinoma (UCC) of the bladder is the fifth most common non-skin malignancy in the western world with approximately 54.300 new cases in the USA per year [1]. In most patients, UCC is superficial at first presentation. After trans-urethral resection (TUR), these patients require repeated long-term surveillance since 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 non-invasive 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 (BTA) and the nuclear matrix protein (NMP22) 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 frequently found throughout the genome. Microsatellite markers are altered in many cancers reflected by loss of heterozygosity (LOH) and microsatellite instability (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].

The group of Sidransky previously showed the feasibility of microsatellite analysis (MA) for non-invasive detection of bladder cancer in urine [14-16]. Subsequently, several independent groups have confirmed the superior sensitivity of MA (75-96%) compared to morphological cytology (13-50%) [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 fibroblast growth factor receptor 3 *(FGFR3)* gene mutations, responsible for several inherited skeletal disorders, have recently been detected 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 to only 7% of ≥pT2G3 tumors [25]. Therefore, the detection of these mutations in urine may provide an additional mode of non-invasive 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.

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 one day prior to 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 two 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 prior to 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 Papanicolaou 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 (germline) DNA, were collected from each patient and stored at +4°C. Within 6 hours after voiding, the urine samples were divided and processed further 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 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) by polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis as described [24,25]. In brief, three regions encompassing activating *FGFR3* mutations previously found 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 0.2x (exon 7) or 1x SSCP-buffer (exons 10 & 15) (10x SSCP-buffer = 0.5M Tris-borate, 1mM 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.

Microsatellite analysis. 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; D4S3038, F= 5'-CTATAGGGGGGTGAAGCAACAG and R= 5'-TGAGAGAATATGGCTATGTGGG. The annealing temperature for these 4 additional markers was 60°C. The polymerase chain reaction (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 allelic-imbalance (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-square 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 non-informative (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, 3 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 these mutations were 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 two 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 (not shown). Yet, the microsatellite alterations on the chromosomal arms 4p, 11q, 17p and 20q were related to higher-stage UCC; P-values (χ-square): 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-values (χ-square): 0.049 and <0.001, respectively. Twenty-eight UCCs had microsatellite alterations at 17p (14/15 tumors staged ≥pT1; 11/28 G2 and 14/15 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 Figure 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.

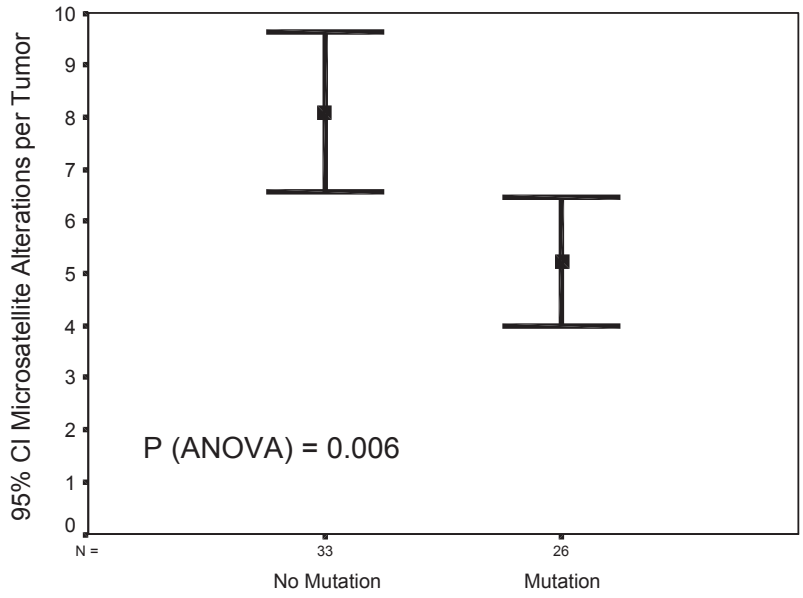
Table 1

Correlation of clinical tumor characteristics with molecular findings in tumor tissue.
The FGFR3 status and the mean number of altered markers at microsatellite analysis are shown for the 59 cases of urothelial cell carcinoma. The clinical tumor variables are primary/recurrent UCC, size, multifocality, stage and grade.

		FGFR3 status			Microsatellite analysis	
		No mutation	Mutation	P (χ-square)	Mean # 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)	
# 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*	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)	

** pT1 in 3, pT2 in 8, pT3 in 2 and pT4 in 1 case(s)*

Figure 1



Correlation FGFR3 mutation and number of altered microsatellite markers in urothelial cell carcinoma. The figure shows the mean number of microsatellite alterations for the 33 tumors without FGFR3 mutation, i.e. 8.1 alterations per tumor (95% CI, 6.6 – 9.6), and for the 26 tumors with a mutation in the FGFR3 gene, i.e. 5.2 alterations per tumor (95% CI, 4.0 – 6.4).

***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*. Fifteen (25%) of the 59 analyzed UCCs had LOH at 4p16.3. Only seven of these 15 also displayed a mutation in the *FGFR3* gene. As a consequence, 19/26 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 recently reported 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 Figure 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, more than 50 leukocytes per microscopic view (magnification 400x) were found (example Figure 2D). Both molecular- (MA and *FGFR3* status) and morphological cytology detected only one 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 44/53 (83%), 11/53 (21%) and 13/51 (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 (15/21; 71%) compared to *FGFR3* wild-type UCC (29/32; 91%), we explored the possibility to combine MA and *FGFR3* mutation screening to enhance the sensitivity. Indeed, we detected three more UCCs by including the *FGFR3* status to the MA of urine. Consequently, the molecular detection of *FGFR3* positive UCC increased from 15/21 to 18/21 and the overall sensitivity of molecular cytology increased to 47/53 (89%). The combination of molecular cytology and morphological cytology did not further enhance the sensitivity or decrease the specificity (not shown).

Legend of Figure 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 microsatellite analysis 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 microsatellite analysis, the altered markers in tumor and/or urine are presented. Loss of the upper allele, loss of the lower allele and microsatellite instability are indicated as shown at the bottom of the figure. No LOH (allelic imbalance (AI) ratio ≥ 0.70) or MSI is indicated by a white box. Non informative 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.

Example 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 microsatellite analysis.

Example B, a pTaG1 lesion, is not detected by microsatellite analysis but is detected by the FGFR3 mutation analysis of the urine.

In example C, a loss of heterozygosity for the marker ACTBP2 was observed in the urine, which was not observed in the tumor.

Example D shows the microsatellite analysis of a bladder-tumor patient with leukocyturia.

Next to multiple losses, example E shows microsatellite instability of tumor and urine for the marker D11S488.

Example F is the opposite of example B. It shows a pTaG1 lesion that is detected by microsatellite analysis but not by FGFR3 mutation analysis of urine.

Figure 2

A

nr. 5/01	**Tumor**	**Urine**
***FGFR3* gene:**		
Exon 7	S249C	S249C
Microsatellite Analysis:		
FGA		
D4S243		
D11S488		
THO		
D9S752		
D9S144		
D17S786		
D17S960		
Clinical Record:	Primary	Cytol.: -
	>2.0 cm	0-10 leuc.
	Solitair	
	pTa G2	
	mid-Ureter	

B

nr. 86/01	**Tumor**	**Urine**
***FGFR3* gene:**		
Exon 7	S249C	S249C
Microsatellite Analysis:		
D9S752		
Clinical Record:	Primary	Cytol.: +
	>2.0 cm	10-50 leuc.
	Solitair	
	pTa G1	
	Right wall	

C

nr. 13/01	**Tumor**	**Urine**
***FGFR3* gene:**		
	wt	wt
Microsatellite Analysis:		
ACTBP2		
FGA		
D16S476		
D18S51		
D4S243		
D9S242		
D16S310		
D17S695		
LPL		
D4S412		
Clinical Record:	Primary	Cytol.: +
	>2.0 cm	0-10 leuc.
	Solitair	
	pTa G3	
	Posterior	

D

nr. 137/02	**Tumor**	**Urine**
***FGFR3* gene:**		
	wt	wt
Microsatellite Analysis:		
ACTBP2		
FGA		
D16S476		
D18S51		
D4S243		
D9S252		
D16S310		
D17S695		
D17S960		
D4S1614		
Clinical Record:	Recurrent	Cytol.: -
	0.5-2.0 cm	>50 leuc.
	Multiple	
	pT2 G3	
	Multiple loc.	

E

nr. 157/01	**Tumor**	**Urine**
***FGFR3* gene:**		
	wt	wt
Microsatellite Analysis:		
ACTBP2		
FGA		
D16S476		
D9S242		
D11S488		
D9S171		
D16S310		
THO		
D9S752		
D17S695		
D9S144		
D20S454		
D17S786		
D4S1614		
Clinical Record:	Primary	Cytol.: -
	0.5-2.0 cm	0-10 leuc.
	Solitair	
	pT1 G2	
	Left wall	

F

nr. 22/02	**Tumor**	**Urine**
***FGFR3* gene:**		
Exon 10	G372C	wt
Microsatellite Analysis:		
ACTBP2		
D16S310		
D4S412		
Clinical Record:	Recurrent	Cytol.: -
	0.5-2.0 cm	0-10 leuc.
	Solitair	
	pTa G1	
	Right wall	

	loss of the lower allele
	loss of the upper allele
	microsatellite instability

Table 2

Molecular and morphological urine cytology for different clinical and molecular tumor-features. Sensitivity (n=53) and specificity (n=15) are shown.

		Molecular Cytology[+]	Morphological Cytology*
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)
No 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**	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)

[+] *combination of Microsatellite Analysis and FGFR3 mutation detection in voided urine.*

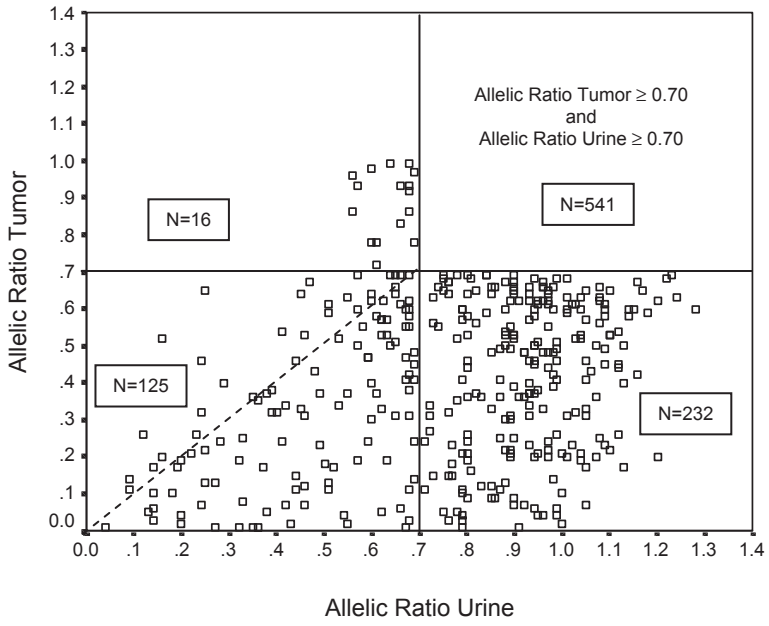
** In 2 cases, cytology diagnosis was not possible.*

*** See footnote Table 1*

Table 2 shows the performance of molecular- and morphological cytology for the different clinical and molecular variables of the tumors. The superior sensitivity of the molecular detection compared to the morphological approach is evident for all clinical- and molecular variables. Furthermore, we determined the specificity of molecular and morphological cytology on the urine samples of fifteen 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, *FGFR3* mutations and morphological cytology diagnosis was negative. Consequently, the specificity of molecular- and morphological cytology was 93% (Table 2). In the one remaining patient, all 3 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 non-invasive 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 Figure 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 16/914 (1.8%) of the informative cases (Figure 3). These data indicate that the urine contained the same genetic aberrations as the analyzed tumor.

Figure 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 urothelial cell carcinoma 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 (Allelic (imbalance) 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 loss of heterozygosity for the same allele. For the samples below the dashed line (N=96), the allelic (imbalance) ratio was higher, i.e. closer to 0.70, in urine as compared to 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, 33/100 (33%), and for patients with *FGFR3* wild type tumors, 92/257 (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 Figure 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 62/132 (47%) in case of multiple tumors and 63/225 (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.

Table 3
Correlation of FGFR3 status and LOH between tumor and urine, and the effect of Leucocyte abundance.

	FGFR3 status		
	Mutation (Tumor)	Same mutation (%) (Urine)	P (χ-square)
Leuc. <50 (N=53)	21	11 (52%)	P=0.033
Leuc. ≥50 (N=6)	5	0 (0%)	
	Microsatellite analysis		
	Microsatellite alterations (Tumor)	Same microsatellite alteration (%) (Urine)	P (χ-square)
Leuc. <50 (N=53)	357	125 (35%)	<0.001
Leuc. ≥50 (N=6)	46	1 (2%)	

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 non-invasive 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 further 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) [25]. Moreover, only 4/28 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 [2, 9, 28], 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]. We here 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% (47/53) by adding the *FGFR3* mutation analysis, without compromising the specificity (93%, 14/15), we advocate the addition of the *FGFR3* mutation analysis, particularly the exons 7 & 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 16/914 (1.8%) of cases for MA (23 markers) and 2/159 (1.3%) for *FGFR3* mutation analyses (exons 7, 10 &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. Since we only analyzed one 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 metachroneous UCC [31]. In the present study, 52% (11/21) of the *FGFR3* mutations and 35% (125/357) 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 further 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, Chistensen *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, Chistensen *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 to 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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5

Surveillance with Microsatellite Analysis of Urine in Bladder Cancer Patients Treated by Radiotherapy

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ABSTRACT

The interpretation of cystoscopy and cytology may be troublesome in bladder cancer patients previously treated by radiotherapy. We evaluated PCR-based molecular cytology by microsatellite analysis (MA) and routine- (RUC) and expert (EUC) urine cytology as modes of surveillance for patients previously treated by radiotherapy with curative intent. Eighty-one voided urine-samples were obtained from 49 patients prior to cystoscopy and subjected to MA, RUC and EUC. During the follow-up period, 6 patients developed a recurrence. Sensitivity of MA, RUC and EUC was 83%, 50% and 33%, respectively. The specificity of MA, RUC and EUC was 93%, 85% and 97%, respectively. Cystoscopy was positive in 15 cases. Therefore, the positive predictive value of cystoscopy remained limited to 40%. Next to recent studies demonstrating a high accuracy for MA in non-irradiated patients, our results indicate that molecular cytology by MA may also be a useful tool to improve the surveillance of bladder cancer patients previously treated by radiotherapy.

INTRODUCTION

Close surveillance and intravesical treatment are the keystones in the management of early stage, superficial bladder cancer.[1,2] On the other hand, the treatment of muscle invasive bladder cancer (i.e. ≥T2) mostly relies on radical cystectomy. For a selected group of patients, bladder sparing strategies, such as trans-urethral resection (TUR) followed by partial cystectomy, radiotherapy or combinations of these have shown to be effective in controlling invasive disease and result in definitive tumor control.[3-6] The patients treated with a bladder preserving strategy also require close and life-long surveillance for a possible recurrence of their disease. Currently, cystoscopy is the standard practice for follow-up of bladder cancer but it remains an uncomfortable procedure for many patients. Although urinary cytology is widely used as non-invasive detection method, its application is largely limited by low sensitivity and operator dependency, especially for low-grade tumors.[7,8] Moreover, cystoscopy as well as cytology are often less reliable in case of previous radiotherapy.[9,10]

Microsatellite markers are altered in many cancers reflected by loss of heterozygosity (LOH) and microsatellite instability (MSI). In bladder cancer, LOH is frequently found on the chromosomal arms 4p, 8p, 9p, 9q, 11p and 17p[11] whereas MSI is less common[12]. The same genetic alterations have also been used to detect these cancer-related features in body fluids.[13] The group of Sidransky has previously shown the feasibility of microsatellite analysis (MA) for the detection of bladder cancer in voided urine.[13-15] Subsequently, several independent groups have recently confirmed the superior sensitivity of MA (75-96%) compared to cytology (13-50%) in various clinical settings.[16-20] However, to our knowledge, no report exists on MA of urine in patients under surveillance after radiotherapy.

In the present study, we evaluated routine urine cytology (RUC), expert urine cytology (EUC) and MA for a cross-section of patients in follow-up who previously received radiotherapy with curative intent.

METHODS

Patients. Forty-nine (38 male, 11 female) patients who regularly attended the Department of Radiation Oncology of the Erasmus MC - Daniel den Hoed Cancer Centre (DHCC) for follow-up were included. The patients were referred to the DHCC after TUR and underwent radiation therapy with curative intent for bladder cancer. Their tumor- and radiation characteristics are shown in Table 1.

Table 1
Tumor- and radiation characteristics of the 49 studied patients.

		No Patients (%)
Stage	T1	11 (22)
	T2	38 (78)
Grade	G2	8 (16)
	G3	41 (84)
Histological type	Transitional Cell Carcinoma	45 (92)
	Adenocarcinoma	2 (4)
	Small Cell Carcinoma	1 (2)
	Squamous Cell Carcinoma	1 (2)
Radiation type	EBRT followed by IRT	40 (82)
	IRT	3 (6)
	EBRT	6 (12)
Total		49 (100)

EBRT=external-beam radiotherapy, IRT=interstitial radiotherapy

The histology slides were reviewed according to the 1973 WHO grading system and the 1997 TNM classification guidelines. In addition, the patients had a CT-scan and chest X-ray, which were negative for (lymph node) metastases. Prior to radiotherapy, six patients had intravesical treatment and one patient had systemic chemotherapy. Three patients with a T1 tumor received interstitial radiotherapy (IRT) 60 Gy and six patients received external-beam radiotherapy (EBRT) 66 (33x2.0) Gy. Most patients (n=40) underwent EBRT 20x2.0 Gy followed by IRT 30 Gy. Three of these patients who were treated before 1990, had IRT via caesium needles. In 37 patients, the IRT was given using the afterloading iridium wire technique as described.[5] The IRT was applied in case of solitary bladder cancer with a limited (<5 cm) surface diameter. Most patients treated by IRT had a partial cystectomy of the original tumor-site. After completion of the radiation therapy, the follow-up was done with cystoscopy and cytology every three months for the first 2 years and every six months thereafter. An intravenous urogram was made every 2 years to monitor the upper urinary tract. The patients local urologist alternated with the DHCC executed this follow-up. At least one follow-up visit per year was performed at the DHCC. The

medical-ethical committee of Erasmus MC approved the study (MEC 168.922/1998/55, AZR 99/01). The patients signed written informed consent prior to study inclusion. The mean age at time of study inclusion was 66,9 years (range, 42-85). Eighty-nine urine samples were obtained. In eight cases, more than 50 leukocytes per microscopic view (magnification x400) were seen. These samples were excluded from further analysis since leukocyte abundance causes false negative results for MA.[19] The remaining 81 urine samples were retrieved from the 49 patients as follows: one, two, three and four samples in 29, 10, 8 and 2 patients, respectively. In case of a positive cystoscopy, TUR was scheduled. Recurrence was defined as histological confirmed cancer at TUR.

Sample collection and DNA extraction. Freshly voided urine samples (15-50 ml) and venous blood (7 ml), as a source of reference (germline) DNA were collected. Venous blood was only collected at the first follow-up visit after study inclusion and stored at -80°C. Within 6 hours after voiding, the urine samples were divided and processed for cytology and MA. The cytological material was stained by standard Papanicolaou staining. The final diagnosis of the cytopathology department was used as result of RUC. The results of the EUC and a leukocyte count were provided by VK-B who reviewed the cytology slides. The urine samples for MA were spun at 2,000 rpm for 6 min. The pellet was rinsed with 10 ml phosphate-buffered saline (PBS) and spun again at 2,000 rpm for 6 min. This pellet was resuspended in 800 µl PBS, brought to a 1.5 ml tube and spun down at 14,000 rpm for 2 min. These tubes were also stored at -80°C. DNA from venous blood was isolated using the QIAamp® DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) and DNA from the urine-pellets was extracted using the QIAamp® Viral RNA kit (Qiagen GmbH, Hilden, Germany). The DNA samples were coded and stored at -30°C.

Microsatellite analysis. 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.[19] These 19 markers are located on 12 chromosomal arms. The polymerase chain reaction (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 allelic-imbalance (AI) ratio between 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 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 was confirmed by a second PCR. The AI ratio closest to 1.0 was used. These analyses were done in a blinded fashion, without

knowledge of the clinical status. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for data documentation and analysis. The sensitivity, specificity and the positive- and negative predictive value were determined for each test in urine.

RESULTS

We have tested 81 urine samples of 49 patients who were under surveillance after radiotherapy with curative intent. The mean time between final irradiation and first urine collection (study inclusion) was 38.9 months (range, 3-172). Twelve urine samples were collected within one year from completion of the radiation therapy. Six recurrences were found at TUR. The stage and grade of these tumors were TaG1, T2G3 and T3G3 in 1, 4 and 1 case(s), respectively. The Tumor and radiation characteristics of the 6 patients who developed a recurrence are depicted in Table 2.

Table 2

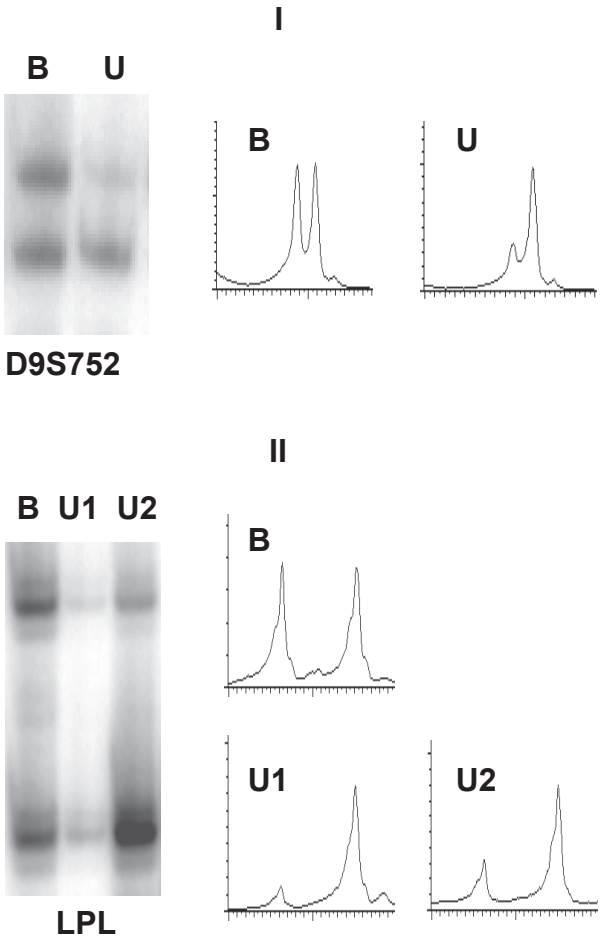
Tumor & radiation characteristics of the 6 patients who developed a recurrence. Initial pT/G refers to the stage and grade of the original tumor that was treated by radiotherapy. The histological types also refer to the original tumor. The time interval was calculated from the initiation of radiotherapy to the date of recurrence.

Case#	Initial pT/G	Recurrent pT/G	Histological type	Radiation type	Time interval (years)
20	pT2/G3	pTa/G1	TCC	EBRT	3.8
27	pT1/G3	pT3/G3	TCC	EBRT	4.7
45	pT2/G3	pT2/G3	TCC	EBRT/IRT	1.8
59	pT2/G3	pT2/G3	Adeno	EBRT/IRT	2.0
60	pT2/G3	pT2/G3	TCC	EBRT/IRT	1.1
70	pT2/G3	pT2/G3	TCC	EBRT/IRT	8.7

TCC=Transitional cell carcinoma, Adeno=Adenocarcinoma, EBRT=External-beam radiotherapy, EBRT/IRT= External-beam radiotherapy followed by Interstitial radiotherapy.

Several representative examples of MA are shown in Figure 1. MSI was not found at all. LOH was found in 5/6 patients with a recurrence (sensitivity: 83%). The urine of the patient with the TaG1 lesion did not show LOH. In the urine of 5 patients with LOH and a recurrence, 16 LOHs were found at MA. In the samples of patients without a recurrence (n=75), 70 MAs were negative (specificity: 93%). In the 5 samples with LOH but without a recurrence, only 6 LOHs were found. No false positive results were found within one year from completion of the radiation therapy.

Figure 1



Two representative examples (roman numerals I and II) of microsatellite analysis in voided urine. The migration patterns after gel electrophoresis are shown on the left side. The notation of the particular microsatellite marker is indicated below. The lanes represent normal, i.e. blood, DNA (B) and urine DNA (U). The corresponding calculation of the allelic-imbalance ratios is shown on the right side. The Allelic Imbalance (AI) ratio was calculated by division of the ratio in urine DNA (U) and the ratio in blood DNA (B). Loss of Heterozygosity (LOH) for a microsatellite marker was scored if the AI ratio was ≥1.43 or <0.70, depending on loss of the upper or lower allele. In example I, the AI ratio was 0.54 (U=0.56/B=1.03). In example II, two urine samples (U1 and U2) of the same patient were tested. The AI ratios were 0.16 and 0.38, respectively. In this case, the microsatellite analysis was already positive (U1) 3 months before biopsy was performed (U2).

RUC and EUC were positive for 3 and 2 of the six recurrences, respectively. A definitive cytological diagnosis was not possible for 4 cases (RUC) and 1 case (EUC). RUC was negative for 60/71 urine samples without a recurrence (specificity: 85%). EUC was negative for 72/74 samples without a recurrence (specificity: 97%). Table 3A shows the sensitivity, specificity and the positive- and negative predictive values for the 3 different tests in urine.

Cystoscopy was positive in 15/81 cases. However, 9/15 TUR-specimens showed no malignancy at histopathological examination; in other words, the

positive predictive value of cystoscopy was limited to 40%. The specificity of cystoscopy for the diagnosis of bladder cancer was 88% (66/75) in the present study. Table 3B shows the relation between the cystoscopical result and the recurrences detected at TUR.

Table 3A

Results of routine urine cytology (RUC), expert urine cytology (EUC) and urinary microsatellite analysis (MA) based on 81 voided urine samples of 49 patients. RC and EC yielded no diagnosis in 4 and 1 case(s), respectively. Absolute numbers are between parentheses.

	%RUC	%EUC	%MA
Sensitivity	50 (3/6)	33 (2/6)	83 (5/6)
Specificity	85 (60/71)	97 (72/74)	93 (70/75)
Pos predictive value	21 (3/14)	50 (2/4)	50 (5/10)
Neg predictive value	95 (60/63)	95 (72/76)	99 (70/71)

Pos=positive, Neg=negative

Table 3B

The number of histologically confirmed recurrences and the results of the 81 cystoscopies performed in 49 patients are shown. The specificity and the positive predictive value of cystoscopy amount to 88% (66/75) and 40% (6/15), respectively. The relative low positive predictive value supports the assumption that the interpretation of cystoscopy in patients who underwent radiotherapy is often troublesome.

	Cystoscopy negative	Cystoscopy positive
No recurrence	66	9
Recurrence	0*	6
Total	66	15

**By definition 0 as the cystoscopical result forms the indication for a biopsy.*

DISCUSSION

Bladder cancer surveillance after radiotherapy is cumbersome and expert opinions are often warranted.[9,10] Even so, the follow-up scheme is more or less the same as for patients with superficial bladder cancer with frequent cystoscopy as the gold standard and cytology as an adjunct. However, a reliable assay on urine may greatly benefit the patients and help the clinicians to determine the strategy for their patients.

We evaluated whether MA of urine was a reliable method for surveillance after radiotherapy. The same urine samples were also examined by cytology, i.e. RUC and EUC. For MA, we reported a sensitivity of 83%, which was comparable to previous observations of our group and others in non-irradiated patients.[13-20] The sensitivity of cytology (50 and 33%) was also in line with these previous studies. However, these results are to be interpreted with caution as only 6 recurrences were documented. Possible reasons for few recurrences in this patient population may be the variable time between the first TUR and the follow-up visit, the selection criteria for brachytherapy and the use of multiple urine samples per patient. In contrast with the low sensitivity, the advantages of urinary

cytology are its high specificity and high negative predictive value.[8,21] However, after irradiation, specificity may lag behind.[9,10] We reported a specificity of 85% for RUC and of 97% for EUC confirming that EUC is required in case of previous radiation therapy, as suggested by others.[10]

A wide variety of biological markers has been developed to address the shortcomings of cytology (low sensitivity, observer variability).[7,8,21] The FDA-approved tests encomprise the bladder tumor antigen (BTA) test, the BTAstat test, the fibrinogen/fibrin degradation products (FDP) assay, the nuclear matrix protein-22 (NMP-22) assay and the UroVysion assay. In general, these are characterized by a higher sensitivity but a lower specificity than cytology.[8,21] On the other hand, MA of urine showed a high specificity (>85%) in several studies[15,17-20] and also showed a higher specificity in comparison with other diagnostic tests in urine[19,20]. However, no study in bladder cancer patients and only one study by Crane et al.[22] who prospectively investigated 18 patients with non-bladder pelvic cancer by the BTAstat test after radiotherapy, have been performed in patients who received radiotherapy. Crane et al.[22] reported positive BTAstat tests in 56% of patients during a median follow-up of 42 weeks indicating that irradiation by itself can cause false-positive BTAstat tests in the first year after radiotherapy. However, we here report a specificity of 93% (70/75) and a negative predictive value of 99% (70/71) for MA. These high percentages indicate that MA may serve as an additional tool for surveillance after radiotherapy for bladder cancer.

Radiation induced genomic instability is a well-known phenomenon.[23] However, the clinical implications of this information for the treatment of cancer with irradiation are not known. Moreover, Boyd et al.[24] found an increase of microsatellite alterations in human somatic cells in culture when they increased the radiation dose. Theoretically, radiation therapy could induce LOH, especially, shortly after the irradiation is given. However, the high specificity and high negative predictive value in the present series do not support this hypothesis. Moreover, the genetic changes that may have been induced by irradiation will only become apparent in a clinical setting if clonal outgrowth of the genetically altered cell in question occurs.

A cystoscopical examination in a patient who received radiotherapy can be difficult.[25] The radiation effects, such as ulceration or reactive epithelial proliferations, frequently accompanied by hematuria, may prompt the urologist to schedule a TUR or a biopsy. In a previous study in 93 non-irradiated patients who were followed after TUR of superficial bladder cancer, we found a specificity of 90% and a positive predictive value of 77% for cystoscopy.[19] The positive predictive value of cystoscopy was only 40% in the present study. This low positive predictive value indeed supports the assumption that the interpretation of cystoscopical findings in patients who underwent radiotherapy is often problematic.

CONCLUSIONS

Like others, we have shown that both cystoscopy and cytology may be cumbersome in bladder cancer patients previously treated with radiation. However, studies on how to improve the follow-up of these patients are very rare. Nevertheless, a reliable method in urine to complement cystoscopy, like EUC or MA, is especially relevant for these patients because the positive predictive value of cystoscopy remains limited after radiotherapy. Here, we show that molecular cytology by MA enables a highly sensitive and specific mode for surveillance. As molecular methods become increasingly available with the introduction of automated techniques, our results suggest that PCR-based molecular urinary cytology may be a useful tool to improve the surveillance of bladder cancer patients including those previously treated by radiotherapy.

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PART III

The Favorable *FGFR3* Mutation in the Prognosis of Bladder Cancer

6

Clinico-pathological Aspects of Superficial Bladder Cancer and the *FGFR3* gene

Based on:
BWG van Rhijn, ThH van der Kwast, AN Vis, WJ Kirkels, F Radvanyi, DK Chopin and EC Zwarthoff. *FGFR3* and *P53* as Molecular Markers in the Diagnosis of Urothelial Cell Carcinoma. Histopathology 2002;41(suppl.2):419-23

ABSTRACT

Urothelial Cell Carcinoma is the fifth most common cancer in humans. For superficial UCC, 70% of the patients develop recurrence(s) and in 10-15% the tumor becomes invasive. Therefore, these patients are intensively monitored by cystoscopy after a trans-urethral resection. In UCC, two types of genetic alterations prevail: multiple losses of heterozygosity pockmark the cancer genome and point mutations are found in the genes for FGFR3 and P53. Mutations in *FGFR3* occur predominantly in UCC whereas *P53* mutations are found in over 50% of human cancers. We here give a short overview of the histopathological and clinical parameters of (superficial) UCC. In addition, some background information on the *FGFR3* gene that is mutated in a high percentage of superficial UCCs, is provided.

CLINICO-PATHOLOGICAL FEATURES OF BLADDER CANCER

Urinary bladder cancer is the fifth most common neoplasm in western society with approximately 54 400 new cases in the USA in 1998.[1] In the Netherlands, approximately 2550 new cases present each year.[2] Histologically, 90-95% of bladder cancer in the western world is urothelial cell carcinoma (UCC). The majority of UCCs are found in the bladder. UCCs of the renal pelvis, ureter and urethra together account for less than 10% of the UCCs. Staging is performed according to the TNM97 system. Clinically, a distinction is made between superficial and invasive UCC. Superficial UCC are neoplasms that do not infiltrate (pTa) or do so superficially (pT1). Patients with solitary pTaG1-2 UCC have a good prognosis (5-year survival around 95%). Invasive bladder cancer has invaded the detrusor muscle. Therapy with curative intent generally consists of cysto(prostat)ectomy provided that the cancer has not yet metastasized. Invasive UCC is a life-threatening disease, as the 5-year survival is about 50%, even after curative surgery.

Carcinoma in situ (CIS) of the urinary tract is a flat lesion, which may occur as an isolated lesion in about 5% of patients with bladder cancer. It may be widespread in the mucosa as a manifestation of field cancerization. CIS is regarded as the precursor of non-papillary pT1 UCC and invasive UCC. The biological behavior of isolated CIS is variable, but ultimately about 50% of the cases show progression.[3] Conversely, CIS is frequently found next to papillary G3 UCC. Thus, CIS can be considered as a precursor lesion of invasive cancer or as a manifestation of intra-epithelial spread of high-grade UCC. The lesions that may be found in association with low-grade papillary neoplasms are flat urothelial hyperplasia and papillary hyperplasia. The cytonuclear features of these lesions do not reveal abnormalities. However, a recent paper described genetic changes in papillary hyperplasia[5] that are similar to those found in papillary UCC, suggesting that this lesion also represents a precursor lesion.

PROGNOSTIC FEATURES OF SUPERFICIAL UCC

Superficial UCCs are generally treated by a trans-urethral resection (TUR) and by intravesical treatment in an adjuvant setting. Although the majority of these superficial UCCs behave rather indolent, their recurrence rate is high, up to 75%, either as a re-growth at the original site or as new lesion(s) elsewhere. This high recurrence rate causes considerable morbidity to this group of patients. Close monitoring of superficial UCC is warranted as, in due time, about 10-15% show progression to invasive UCC.[6] Currently it is routine to monitor our patients with superficial UCC by urethro-cystoscopy (UCS) every 3-4 months for the first two years after a TUR and subsequently at a reduced interval. This procedure is associated with anxiety and physical complaints, including dysuria and cystitis in about 10%. Therefore, much research efforts are undertaken to reduce the UCS frequency for follow up by applying new cancer markers in urine or by defining a subset of patients with superficial UCC and a favorable recurrence frequency.

A major problem to assess prognosis is the inter-observer variation among pathologists.[7] This has obviously stimulated the search for more objective molecular markers to distinguish aggressive from indolent superficial UCC. Multiplicity as a clinical parameter is also of help to predict the chance for future recurrence(s).[6] In addition, presence of CIS is a very unfavorable sign that is associated with a high likelihood of UCC progression.[8] In patients with an upper urinary tact UCC, a considerable risk for future bladder recurrence(s) exists. On the other hand, patients with a single, small-sized, Grade 1 UCC at first presentation are at a low risk for subsequent lesion(s). Molecular indices which have been shown to be associated with a higher risk for recurrence(s) include the *P53* mutation, DNA-aneuploidy and a high expression of the proliferation marker MIB-1.[9,10] A drawback of these markers is that they are relatively rare in superficial UCC, which implies that their absence does not define a subset of patients who have a lower risk for recurrence. Moreover, these markers were found to be strongly correlated with the conventional parameters grade and stage.[9] Recently, the *FGFR3* mutation has been identified as a favorable molecular marker for UCC.[11-13]

FGFR3 MUTATIONS AND BLADDER CANCER

The *fibroblast growth factor receptor 3 (FGFR3)* is a glycoprotein composed of three extracellular immunoglobulin-like domains, a transmembrane domain and a split tyrosine-kinase domain.[14] Ligand binding induces FGFR dimerization, resulting in autophosphorylation of the kinase domain and interaction with and phosphorylation of effector signaling proteins.[15] FGFR3 belongs to a family of four highly conserved and closely related cell membrane associated receptors (FGFR1-4). The FGFRs are involved in cell signaling pathways regulating embryonic growth, cell proliferation, angiogenesis, development and differentiation.[15] Point mutations in specific domains of FGFR3 are associated with autosomal dominant dwarfism and craniosynostosis

syndromes that sometimes coincide with dermatological disorders.[16] Several reports have demonstrated that these mutations lead to constitutive activation of the receptor and that specific mutations lead to different levels of constitutive FGFR3 activation. Probably because of these different activation levels of the receptor, complex phenotype-genotype correlations exist.[16] For example: achondroplasia (the most common form of genetic dwarfism) entails a relative normal life span whereas thanatophoric dysplasia is considered a lethal form of skeletal dysplasia.

Besides the inhibitory role of FGFR3 mutations in skeletal disorders, an oncogenic role for FGFR3 in human cancer has emerged. Indeed, somatic activating mutations in *FGFR3* have been reported in multiple myeloma and, more recently, in two epithelial malignancies, i.e. bladder- and cervix carcinomas.[16-18] *FGFR3* mutations are rare in multiple myeloma and cervix carcinomas, whereas their high incidence in bladder carcinomas (74% of non-invasive papillary tumors) suggests that the constitutive activation of FGFR3 is an important event for bladder tumorigenesis.[11,12] It has been shown that mutated *FGFR3* can transform NIH 3T3 cells and act as an oncogene.[19] Since the *FGFR3* gene is expressed in bladder cancer and normal urothelium,[17] it is likely that the mutant gene has an oncogenic role in bladder cancer pathogenesis.

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7

The Fibroblast Growth Factor Receptor 3 (*FGFR3*) Mutation is a Strong Indicator of Superficial Bladder Cancer with Low Recurrence Rate

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ABSTRACT

We analyzed the possible prognostic value of the recently discovered fibroblast growth factor receptor 3 *(FGFR3)* mutations in bladder cancer. A *FGFR3* mutation was found in 34/53 pTaG1/2 bladder cancers, whereas none of the 19 higher staged tumors had a mutation ($P<0.0001$). In 57 patients with superficial disease followed prospectively by cystoscopy for 12 months, 14 of 23 patients in the wild type *FGFR3* group developed recurrent bladder cancer compared to only 7 of 34 patients in the mutant group ($P=0.004$). The recurrence rate per year was 0.24 for the *FGFR3* mutant tumors and 1.12 for tumors with a wild type *FGFR3* gene. In addition, *FGFR3* mutation status was when compared to stage and grade the strongest predictor of recurrence ($P=0.008$). This is the first mutation in bladder cancer that selectively identifies patients with favorable disease characteristics. Our results suggest that the frequency of cystoscopic examinations can be reduced considerably in patients with *FGFR3* positive tumors.

INTRODUCTION

Urinary bladder cancer is the fifth most common neoplasm in western society with approximately 54 400 new cases in the United States per year.[1] In the majority of patients bladder cancer is superficial (i.e. pTa-pT1) at first presentation. After trans-urethral resection (TUR) of superficial bladder cancer, patients are monitored by cystoscopy at regular intervals since the recurrence rate of superficial bladder cancer is up to 70%.[2-4] Progression to invasive disease occurs in around 15-20% of patients.[2,3] Clinical and histopathological factors for prediction of tumor recurrence and progression of bladder cancer have been studied extensively.[2-4] Especially tumor grade, stage and recurrence rate are important. In addition, it appeared that mutations in the tumor suppressor genes *TP53* and retinoblastoma (*RB*) are of additional value to assess aggressive tumor behavior.[5] The fibroblast growth factor receptor 3 (FGFR3) is a glycoprotein composed of three extracellular immunoglobulin-like domains, a transmembrane domain and a split tyrosine-kinase domain. Several reports have shown that constitutive activation of the *FGFR3* gene by specific point mutations leads to congenital anomalies such as achondroplasia and thanatophoric dysplasia.[6,7] A frequent t(4;14)(p16.3;q32.3) chromosomal translocation with the breakpoint near *FGFR3* in multiple myelomas (MM) suggested an oncogenic role for the *FGFR3* gene.[8] However, an activating mutation in the *FGFR3* gene occurred rarely in MM.[9] The same missense mutations (R248C, S249C, G372C and K652E) that were observed in thanatophoric dysplasia were recently found in 9 of 26 bladder carcinomas and 3 of 12 cervix carcinomas.[10] It has been shown that mutated *FGFR3* can transform NIH 3T3 cells when targeted to the cell membrane.[11] Since the *FGFR3* gene is expressed in bladder cancer and normal urothelium[10], it is likely that the mutant gene has an oncogenic role in bladder

cancer pathogenesis. The present study assesses the possible prognostic value of the *FGFR3* mutation in bladder cancer.

MATERIAL AND METHODS

Patients. Seventy-two consecutive patients who underwent TUR at the University Hospital Rotterdam in 1998-1999 were entered into this study. The patients were not selected on any clinical or other parameter. All patients signed written informed consent. The medical ethical committee of the Erasmus University and the University Hospital Rotterdam approved the study (MEC 168.922/1998/55). Median age at time of diagnosis was 65.4 years (range, 30-89), male/female ratio was 4.2:1. We examined patient's history and obtained 12 months clinical follow-up on a prospective basis, including a cystoscopic examination every 3 months, from all patients that were diagnosed with superficial bladder cancer at time of *FGFR3* mutation analysis. Grading of cancers was performed according to the WHO classification and staging according to the 1997 TNM classification guidelines (UICC 1997). A recurrence was defined as the presence of histologically proven bladder cancer at a positive cystoscopy following a complete previous TUR. The recurrence rate per year was defined as the number of recurrences divided by the total number of months of follow-up. The result was then multiplied by 12. Hence, the recurrence rate takes into account the clinical course of patients during a longer interval and not only the time to first recurrence.

Sample collection and DNA extraction. The bladder tumors were subjected to careful microdissection after confirmation of the histopathological diagnosis. Venous blood (7 ml) was obtained from every patient to be used as control. DNA from paraffin embedded, formalin fixed neoplastic tissue was extracted using Dneasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to enclosed protocol. DNA from venous blood was isolated using one step DNAzol BD Reagent (Life Technologies, Inc. [Gibco BRL], Grand Island, NY). We checked the accuracy of the microdissection procedure by loss of heterozygosity (LOH) analysis in 18 of the 19 patients with ≥ pT1 tumors comparing the allele intensities of normal and tumor DNA of the same samples that were used for the *FGFR3* mutation analysis. A 50% reduction of allele intensity was considered LOH. We used 23 microsatellite markers on 9 different chromosomes as described before.[12] Every LOH was confirmed in a second PCR.

***FGFR3* mutation analysis.** *FGFR3* mutation analysis was performed by polymerase chain reaction (PCR) - single strand conformation analysis (SSCA) on tumor- and control blood DNA of all patients. Three regions of interest containing the 4 previously identified mutations were amplified by PCR. The primer sequences were the same as used by Capellen et al. (10). The following primer pairs were used: exon 7, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and 5'-TGTGCGTCACTGTACACCTTGCAG-3'; exon 10, 5'-CAACGCCCATGTCTTTG-

CAG-3' and 5'-CGGGAAGCGGGAGATCTTG-3'; exon 15, 5'-GACCGAGGAC-AACGTGATG-3' and 5'-GTGTGGGAAGGCGGTGTTG-3'. All tumors with an aberrant band at SSCA were sequenced with T7 Sequenase v2.0 (Amersham life Science, Inc., Cleveland, OH) on both strands to check the identity of the mutations. Analysis of all samples was carried out in a blinded fashion, without knowledge of clinical or histopathological status.

Statistical analysis. Statistical package for social sciences 8.0 (SPSS Inc., Chicago, IL) and StatXact version 2 (Cytel Software Corporation, Cambridge, MA) computer software were used for data documentation and analysis. Fisher's exact test was used to analyze the *FGFR3* mutation in relation to stage, grade, highest stage and prediction of tumor recurrence. Logistic regression analysis with the backward elimination method was used for comparison of variables to predict recurrence. All P-values are two sided. Statistical significance was assumed if $P<0.05$.

RESULTS

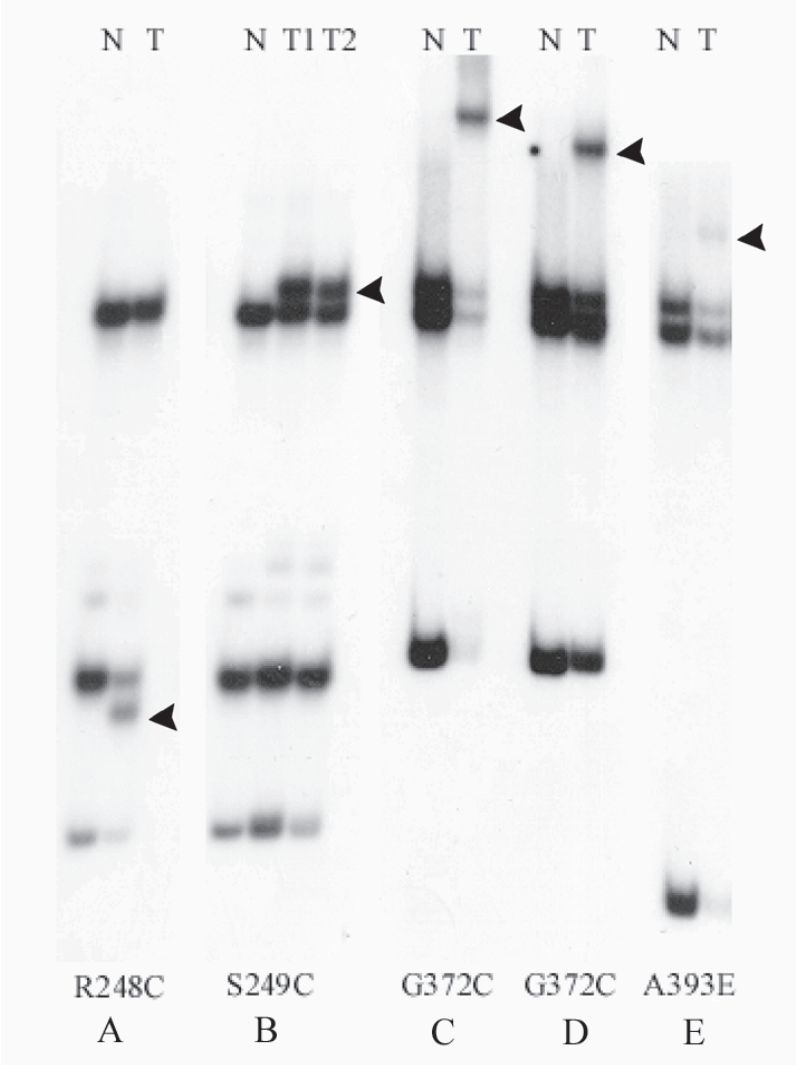
We performed a mutation analysis of the *FGFR3* gene in a group of 72 bladder carcinomas from 72 consecutive patients. Thirty-seven patients were diagnosed with bladder cancer for the first time. All the 72 bladder cancers were transitional cell carcinomas, except for one squamous cell carcinoma that was pT2, Grade 3.

SSCA detected aberrant bands (Figure 1) in DNA samples from 34 tumors but not in DNA from corresponding venous blood, indicating the somatic nature of *FGFR3* mutations in bladder cancer. Thirty mutations concerned the already described codon 249 mutation (S249C). Codon 248 (R248C) and 372 (G372C) mutations were found once and twice, respectively (Figure 1A,C,D). An additional mutation, not observed in bladder cancer before, was also detected (Figure 1E). DNA sequence analysis revealed the point mutation leading to A393E, previously known in a hereditary skeletal syndrome.[13] The two mutations, R248C and S249C, can be found in one amplification region. This region represented 91% of the observed *FGFR3* mutations in our group of bladder cancers. The identity of all mutations was confirmed by DNA sequence analysis. Five samples from blood DNA were also sequenced as negative controls. No mutations were found in these samples. Figure 1 shows that the 5 detected mutations can easily be identified by SSCA.

All 34 mutations were found in the 53 pTa bladder cancers (64%), whereas none of the 4 pT1 and 15 higher staged tumors had a mutation in the gene (Table 1A). Furthermore, all the tumors with a mutation in the *FGFR3* gene were Grade 1 or 2. The mutation did not occur in 19 Grade 3 tumors ($P<0.0001$). The stages of these 19 G3 tumors were pTa in 3, pT1 in 1 and ≥pT2 in 15 cases, respectively. When we compared the patient's history (median history of 5.4 years, range 0.8-26.9 years) with histopathological and *FGFR3* mutation status, we observed that patients with only a pTa history, had a mutation in 33/45 (73%)

cases. In patients whose history revealed at least 1 pT1 tumor, the *FGFR3* mutation occurred in only 1/12 (8%) tumors (P<0.0001, Table 1B). These results suggest that the *FGFR3* mutations are linked with a disease course in which lower staged (i.e. pTa) tumors prevail.

Figure 1



PCR-SSCA of the FGFR3 gene. The figure shows examples of the FGFR3 gene mutations in our population. Paired lanes are normal blood DNA (N) and tumor DNA (T) from the same patient. Solid arrowheads indicate mutations leading to R248C (patient A), S249C (patient B), G372C (patient C), G372C (patient D) and A393E (patient E). In patient B, the same activating point mutation (S249C) was found in the original (T1) and the recurrent tumor (T2). In patient C, an additional silent mutation (G to T transition) at nucleotide position 1113, adjacent to codon 372, was observed. The codon and nucleotide numbering corresponds to the cDNA open reading frame of the FGFR3b isoform. This isoform is expressed in epithelium and contains two aminoacids more than the FGFR3c isoform expressed in bone.

Table 1A
Relation between the tumor stage and the FGFR3 mutation status (n=72).

		FGFR3 mutation analysis		
		Wild Type	Mutation	P[a]
Stage	pTa	19	34	<0.0001
	pT1	4	0	
	≥pT2	15	0	
Total		38	34	

[a] *P values were determined by two-sided Fisher's exact test calculated for 3x2 tables.*

Table 1B
Relation between the highest stage throughout patient's history and the FGFR3 mutation status (n=72).

		FGFR3 mutation analysis		
		Wild Type	Mutation	P[a]
Highest Stage	Only pTa	12	33	<0.0001
	≥ once pT1	11	1	
	≥ once pT2	15	0	
Total		38	34	

[a] *P values were determined by two-sided Fisher's exact test calculated for 3x2 tables.*

To further investigate the link between *FGFR3* mutations and stage, we performed a prospective follow-up analysis. Twelve months follow-up after mutation analysis revealed that bladder cancer recurrence was far more frequent in the group of patients whose initial tumors were wild type with respect to the *FGFR3* gene (P=0.004, Table 2). It should be noted that only patients (n=57) diagnosed with superficial bladder cancer (i.e. pTa, pT1) at time of mutation analysis were included in this follow up analysis since patients with invasive tumors underwent cystectomy or radiotherapy. Moreover, in logistic regression analysis with the backward elimination method, using recurrence as dependent variable and tumor stage, grade, highest stage and *FGFR3* mutation status as independent variables, the *FGFR3* mutation status remained as the only significant (P=0.008) predictor of recurrence.

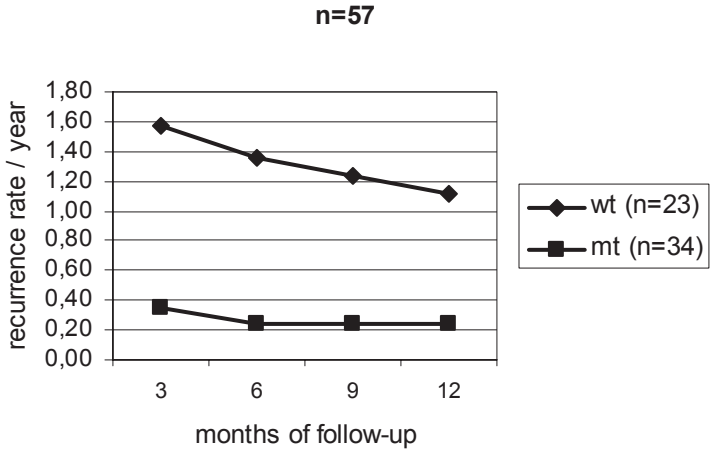
Table 2
Relation between disease recurrence within 12 months in superficial (i.e. pTa, pT1) bladder cancer patients and the FGFR3 mutation status (n=57).

		FGFR3 mutation analysis		
		Wild Type	Mutation	P[a]
12 months follow-up	Recurrence-free	9	27	=0.004
	Recurrence	14	7	
Total		23	34	

[a] *P value was determined by two-sided Fisher's exact test calculated for a 2x2 table.*

We also determined the number of recurrent tumors per patient. In 14 of the 23 patients that were wild type for the *FGFR3* gene, 24 TURs revealed recurrent bladder cancer during the 12 months of follow-up. In 17 of the removed tumors *FGFR3* mutation analysis was performed. No mutations were found. Two patients, both with a wild type *FGFR3* gene, already progressed to invasive (i.e. ≥pT2) disease after 3 months. They were removed from subsequent cystoscopical follow-up. On the other hand, in 7 of the 34 patients with a mutation in their initial tumor, only 8 TURs revealed recurrent bladder cancer (pTaG1 6x and pTaG2 2x). All 8 analyzed tumors retained the *FGFR3* mutation that was initially observed. Consequently, 24 of 86 (28%) cystoscopies were positive in the *FGFR3* wild type group, resulting in a recurrence rate per year of 1.12. In the *FGFR3* mutant group, only 8 of 136 (6%) cystoscopies were positive after 12 months of follow-up, resulting in a recurrence rate per year of 0.24. The recurrence rate per year was calculated at the 3, 6, 9 and 12 month's cystoscopical evaluation points (Figure 2). We also performed a subset analysis of primary and recurrent superficial tumors. The recurrence rates were higher shortly after TUR (3 months) for both patientgroups (Figure 2). The recurrence rates per year were similar if the patients were divided into cases with primary and recurrent cancers (results not shown). In addition, we observed a difference in mutation frequencies between primary and recurrent superficial tumors: where 18/25 (72%) of primary superficial tumors had a mutation in the *FGFR3* gene, this was only 16/32 (50%) for the recurrent tumors.

Figure 2



Recurrence rate of superficial bladder cancer for patients with wild type and mutant tumors. The recurrence rate per year was calculated at the 3, 6, 9 and 12 months cystoscopical evaluation points. After 12 months, 24 of 86 (28%) cystoscopies were positive in the FGFR3 wild type group. This was only 8 of 136 (6%) in the FGFR3 mutant group. wt = wild type FGFR3 gene; mt = mutated FGFR3 gene; n = number of patients who entered cystoscopical follow-up.

DISCUSSION

Bladder cancer is a common type of cancer that has a high incidence, but, more importantly, requires major clinical attention after initial treatment. Nowadays patients are monitored intensively by cystoscopy for possible recurrence. Standard practice following TUR of bladder cancer is to screen patients with a cystoscopic examination every 3-4 months for 2 years and every 6-12 months thereafter. Based on incidence numbers and recurrence rate[1-4], it can be calculated that more than half a million cystoscopies per year are performed in the United States alone.

Our results show that *FGFR3* mutations occur in nearly 50% of bladder cancers. The mutations were exclusively observed in superficial tumors and absent from invasive carcinomas. Moreover, bladder cancer recurrence rates were dramatically lower for tumors with a mutant *FGFR3* gene. We therefore conclude that the *FGFR3* mutations identify a large cohort of bladder cancer patients with favorable disease characteristics. As a consequence, molecular *FGFR3* mutation analysis represents a novel, technically simple and potentially powerful tool for adjustment of clinical management in bladder cancer. Nowadays, all patients undergo frequent cystoscopy to monitor their disease. Our results suggest that the frequency of this uncomfortable, invasive and expensive diagnostic procedure can be reduced considerably in patients with *FGFR3* positive tumors.

We observed a difference in mutation frequencies between primary and recurrent superficial tumors in our study. It has been noted by others that patients who already have recurrent disease continue to develop recurrences more often than patients with a primary tumor.[2,4] We suggest that this difference in mutation percentage is caused by the fact that patients who are cured after a single TUR, approximately 30%, do by definition not take part in the group of patients with recurrent disease. Combining these observations, we feel that it is likely that the group of patients with primary bladder cancer containing a *FGFR3* mutation encompasses patients who will not develop a recurrence at all. Thus, the *FGFR3* mutation itself, being an indicator of superficial bladder cancer with a low recurrence rate, is at least part of the explanation of the differences between these groups. Capellen et al.[10] were the first to report on somatic *FGFR3* mutations in bladder cancer. They found 9 mutations in 26 tumors. Three of the 9 mutations occurred in invasive (i.e. ≥pT2) tumors. The stage distribution of the wild type tumors was not given in their study. In contrast, we found no mutations in invasive tumors. In addition, the correlation between the presence of a mutation and low stage was highly significant in our study. To ensure that no mutations were missed by contamination of benign cells in the invasive tumors, we also performed LOH analysis on these DNA samples. Multiple allelic losses were observed in 17/18 evaluable tumors (results not shown). These losses sustained that the DNA samples used for mutation and LOH analysis indeed contained mainly DNA derived from tumor cells. Thus, the apparent discrepancy

between our study and that of Capellen et al.[10] remains difficult to explain. It may be due to the relatively small number of tumors examined by Capellen et al.[10]

The finding that tumors with *FGFR3* mutations are less likely to lead to recurrences can be explained by the hypothesis that such tumors shed cells with a lower frequency than those tumors that do not carry a *FGFR3* mutation. In the 7 patients with recurrence in the mutant *FGFR3* group, 6/8 tumors occurred at the same region in the bladder and 6/8 recurrent lesions were single growths. In contrast, in most of the 14 patients who had recurrence in the non-mutant group, multiple tumors were found and these tumors occupied several different sites in the bladder. These preliminary data indeed suggest that superficial bladder tumors without a *FGFR3* mutation shed cells more easily and/or that these cells are better equipped to reimplant into the bladder epithelium. In addition, it may be possible that the *FGFR3* mutated tumors proliferate not as fast as the non-mutated tumors. Further experiments are required to test these hypotheses.

Molecular markers for bladder cancer may provide information to be used in clinical decision making. The incidence of *TP53* gene mutations and an altered expression of the retinoblastoma gene product (*RB*) appeared to be much higher in invasive, high-grade bladder cancers than in superficial low-grade ones.[14,15] These reports suggest that these molecular markers are relatively late events in disease pathogenesis and identify tumors with aggressive biological behavior. The clinical value of these 2 tumor suppressor genes is of importance to patients presenting with invasive bladder cancer [15,16] and for prediction of progression in patients with high-grade superficial bladder cancer. Barton Grossman et al. [5] advocated stratification of pT1 bladder cancer patients based on *P53* and *RB* status. Their results suggest that patients with normal protein expression for both genes can be managed conservatively, whereas patients with alterations in one and particularly both genes require more aggressive treatment to prevent progression to invasive disease. In contrast, chromosome 9q deletions, determined by LOH analysis, are found with similar frequency in superficial and invasive bladder cancer[17,18] and therefore can not be used for prognostic purposes. The *FGFR3* gene is the first gene identified in bladder cancer to be mutated selectively in those cancers that are characterized by favorable clinical parameters. Future studies should determine whether a combination of *TP53*, *RB* and *FGFR3* gene analyses could lead to a more accurate prediction of the disease course with regard to recurrence and progression. Furthermore, treatment strategies may also be determined based on these molecular markers.

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8

Frequent *FGFR3* Mutations in Papillary Non-Invasive Bladder (pTa) Tumors

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ABSTRACT

We recently identified activating mutations of *fibroblast growth factor receptor 3 (FGFR3)* in bladder carcinoma. In this study we assessed the incidence of *FGFR3* mutations in a series of 132 bladder carcinomas: 20 carcinoma *in situ* (CIS), 50 pTa, 19 pT1, and 43 pT2-4. All 48 mutations identified were identical to the germinal activating mutations that cause thanatophoric dysplasia, a lethal form of dwarfism. The S249C mutation, found in 33 of the 48 mutated tumors (69%), was the most common. The frequency of mutations was higher in pTA Tumors (37 of 50, 74%) than in CIS (0 of 20, 0%; *P*<0.0001), pT1 (4 of 19, 21%; *P*<0.0001) and pT2-4 tumors (7 of 43, 16%; *P*<0.0001). *FGFR3* mutations were detected in 27 of 32 (84%) G1, 16 of 29 (55%) G2, and 5 of 71 (7%) G3 tumors. This association between *FGFR3* mutations and low grade was highly significant (P<0.0001). *FGFR3* is the first gene to be found to be mutated at a high frequency in pTa tumors. The absence of *FGFR3* mutations in CIS and the low frequency of *FGFR3* mutations in pT1 and pT2-4 tumors are consistent with the model of bladder tumor progression in which the most common precursor of pT1 and pT2-4 tumors is CIS.

INTRODUCTION

Bladder cancer is the fourth most common malignancy in men and the ninth most common in women in the Western world. In these countries, more than 90% of bladder tumors are urothelial carcinomas. At the time of initial diagnosis, approximately 80% of urothelial carcinomas are confined to the epithelium (pTa, CIS) or lamina propria (pT1), whereas the remaining 20% invade the muscularis propria (pT2, pT3, pT4). pTa lesions, the most common form of bladder carcinoma, are papillary tumors. Carcinoma *in situ* (CIS) are flat, cytologically high grade carcinomas. Primary isolated CIS is a very rare entity, CIS being more commonly associated with other malignant bladders lesions.[1]

Clinical evidences and molecular studies suggest that there are two pathways in bladder carcinogenesis responsible for generating two types of urothelium-confined tumors (pTa and CIS) with very distinctive behavior.[1-6] pTa tumors are associated with a high rate of recurrence (50-75%) but low probability (<5%) of progression to lamina propria-invasive (pT1) and muscle-invasive (pT2-4) tumors. CIS may be the most common precursor to invasive bladder cancer because CIS shows a strong tendency to progress (40-50%), and because most muscle-invasive lesions arise with no history of a pTa precursor lesion. This clinical evidence is supported by various molecular studies showing that CIS and invasive tumors have many genetic alterations in common, such as specific chromosomal deletions and a high frequency of *p53* mutations.[2,7]

In our search for new markers of carcinoma progression, we recently reported specific missense mutations in a gene encoding a growth factor receptor, fibroblast growth factor receptor 3 (FGFR3), in a series of 26 bladder and 12 cervical tumors.[8] FGFR3 belongs to a family of structurally related

tyrosine kinase receptors encoded by four different genes (*FGFR1-4*). These receptors are glycoproteins composed of two or three extracellular immunoglobulin (Ig)-like domains, a hydrophobic transmembrane domain, and a cytoplasmic region that contains the tyrosine kinase domain. Binding to members of the fibroblast growth factor family induces FGFR dimerization, resulting in autophosphorylation of the kinase domain and interaction with and phosphorylation of effector signaling proteins. Alternative mRNA splicing mechanisms generate many different receptor isoforms.[9] Isoforms FGFR3b and FGFR3c result from a mutually exclusive splicing event in which the second half of the juxtamembrane Ig-like domain of FGFR3 is encoded by either the 150 nucleotides of exon 8 or the 144 nucleotides of exon 9.[10] FGFR3b and FGFR3c have different tissue distributions; for example, FGFR3b is the main form in epithelial cells, whereas FGFR3c is the form found in chondrocytes.[11] Specific point mutations in various domains of *FGFR3* are associated with autosomal dominant human skeletal disorders such as hypochondroplasia, achondroplasia, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) and thanatophoric dysplasia.[12,13] Several reports have demonstrated that these mutations lead to constitutive activation of the receptor.[12-14] The identification in multiple myeloma and, more recently, in bladder and cervical carcinomas of somatic mutations of *FGFR3* identical to the activating mutations responsible for thanatophoric dysplasia and SADDAN [8,15-17] suggested that FGFR3 plays an oncogenic role for FGFR3.

We investigated the involvement of *FGFR3* in bladder carcinogenesis by assessing the incidence of *FGFR3* mutations in a series of 132 bladder tumors of various stages and grades. The frequency of *FGFR3* mutations (74%) in non-invasive papillary pTa tumors shows that *FGFR3* is a major oncogene in this, the most common form of bladder carcinoma. This high frequency of *FGFR3* mutations in pTa tumors contrasts with the absence of *FGFR3* mutations in carcinoma *in situ* and the low percentage of mutations in pT1 and pT2-4 tumors. These data are consistent with the existence of two pathways of progression in bladder cancer.

MATERIAL AND METHODS

Tumor samples. Tumors were staged according to the TNM classification[18] and graded according to criteria recommended by the World Health Organisation.[19] All slides were reviewed by two pathologists (CB, MPB). For cases where lamina propria invasion was unclear, we performed immunohistochemical analysis with an anti-cytokeratin antibody. The bladder tumor series consisted of 20 carcinoma *in situ* (CIS), 50 pTa, 19 pT1, 14 pT2, 20 pT3 and 9 pT4 tumors; 32 were grade G1, 29 grade G2, and 71 grade G3. The series of 112 pTa and pT1-pT4 tumors were obtained from 112 patients. The 20 CIS were obtained from an additional 17 patients. Nine CIS occurred as isolated lesions; eleven were associated with pTa or pT1-T4 tumors. All of the pTa and

pT1-pT4 tumors and 10 of the CIS were obtained from patients diagnosed at the Henri Mondor Hospital (Créteil, France).The 10 remaining CIS were obtained from patients diagnosed at the Rotterdam University hospital (Rotterdam, The Netherlands. DNA from pTa and pT1-pT4 tumors was extracted from samples frozen immediately in liquid nitrogen as previously described[20] or from formalin-fixed, paraffin-embedded tissue, using the tissue DNA extraction kit from Qiagen (Courtaboeuf, France). All samples retained for this analysis contained more than 80% tumors cells, as assessed by histological examination. For CIS, tumor cells were isolated from paraffin-embedded sections by laser-assisted microdissection, using the PALM Laser-Microbeam System (PALM, Wolfratshaussen, Germany) and DNA was extracted using the Qiagen kit. Normal DNA samples from peripheral blood were available from 45 patients.

***FGFR3* mutation analysis.** Mutations were screened by single-strand conformation polymorphism (SSCP) in four regions of the *FGFR3* gene, located in exons 7, 10, 15 and 19. These four regions contain the *FGFR3* point mutations previously identified in thanatophoric dysplasia, SADDAN, achondroplasia, Crouzon syndrome with acanthosis nigricans, multiple myeloma and bladder and cervical carcinomas.[8,12,13]

For most of the samples (all pTa and pT1-pT4 tumors and 16 CIS), a single round of PCR was carried out, using the following primer pairs: exon 7, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and 5'-TGTGCGTCACTGTACACCTT-GCAG-3'; exon 10, 5'-CAACGCCCATGTCTTTGCAG-3' and 5'-CGGGAAGCG-GGAGATCTTG-3'; exon 15, 5'-GACCGAGGACAACGTGATG-3' and 5'-GTGTG-GGAAGGCGGTGTTG-3'; exon 19, 5'-TGTCGGCGCCTTTCGAGCAGTA-3' and 5'-AGCAGCAGGGTGGGCTGCTA-3'. PCR was performed in a final volume of 50µl containing 50 ng genomic DNA or 1/20th of the purified DNA from microdissected samples, 100 µmol/L each of dATP, dCTP, dTTP and dGTP, 1 µmol/L forward and reverse primers, 1X of amplification buffer provided with the polymerase, 1 µCi [α33P]dATP. The *Taq* polymerase (1 U; Finnzymes Oy, Espoo, Finland) was added after heating the reaction mixture for 5 min at 95°C. The mixture was then subjected to 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, 20 seconds.

A two stage seminested PCR was used to amplify DNA from 4 CIS that gave a weak signal in single-round PCR. For the first round of the seminested PCR we used the following PCR primers: exon 7, 5'-AGTGGCGGTGGTGGT-GAG-GGAG-3' and 5'-TGTGCGTCACTGTACACCTT-3'; exon 10, 5'-GCCAGGC-CAGGCCTCAAC-3' and 5'-CTTGAGCGGGAAGCGGGAGATCTTG-3'; exon 15, 5'-TGGTGACCGAGGACAACGTGATG-3' and 5'-CTCTGGTGAGTGTAGACT-CG-3'. For The second round of the seminested we used the following PCR primers: for exon 7, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and 5'-TGTGCGT-CACTGTACACCTTGCAG-3'; exon 10 5'-CCTCAACGCCCATGTCTTTTCAGC-3' and 5'-CTTGACGGGAAGCGGGAGATCTTG-3'; exon 15, 5'-TGGTGACCGAGG-ACAACGTGATG-3' and 5'-AGGGTGTGGGAAGGCGGTGTTG-3'. First round

PCR conditions were the same as for single-round PCR, except that no radioactive isotope was included in the reaction and only 15 cycles were performed. The first-round product (1 µl) was subjected to a second round of PCR comprising of 35 cycles with conditions identical to those for single-round PCR except that the annealing temperature was 68°C.

Aliquots of the labeled PCR products were subjected to electrophoresis in non-denaturing (MDE)/8.5% glycerol gels at room temperature for 13 hours. Samples displaying a mobility shift in SSCP-analysis were further analyzed by direct bidirectional DNA sequencing. The PCR products were sequenced using the following primers: exon 7, 5'-AGTGGCGGTGGTGGTGAGGGAG-3' and 5'-CAGCACCGCCGTCTGGTTGG-3'; exon 10, 5'-CAACGCCCATGTCTTTGCAG-3' and 5'-GAGCCCAGGCCTTTCTTGG-3'; exon 15, 5'-AGGACAACGTGATG-AAG-ATCG-3' and 5'-GTGTGGGAAGGCGGTGTTG-3'; exon 19, 5'-TGTCGGC-GCCTTTCGAGCAGTA-3' and 5'-TGCTAGGGACCCCTCACATT-3'. Matched normal DNA, if available, was sequenced on both strands to demonstrate the somatic nature of these mutations.

RESULTS

Identification of *FGFR3* point mutations. We used SSCP and sequencing to analyze the regions of *FGFR3* harboring the point mutations found in multiple myelomas and in skeletal disorders, in a series of 132 bladder carcinoma samples. We detected 5 different single-nucleotide substitutions in 48 of 132 bladder carcinomas (Table I).

Table 1

Point mutations of the FGFR3 gene in bladder carcinomas

Codon	Nt position	Exon	Mutation	Predicted effect	Number of tumors
248	742	7	CGC → TGC	Arg → Cys	8
249	746	7	TCC → TGC	Ser → Cys	33
372 (370)	1114	10	GGC → TGC	Gly → Cys	3
375 (373)	1124	10	TAT → TGT	Tyr → Cys	2
652 (650)	1954	15	AAG → GAG	Lys → Glu	2

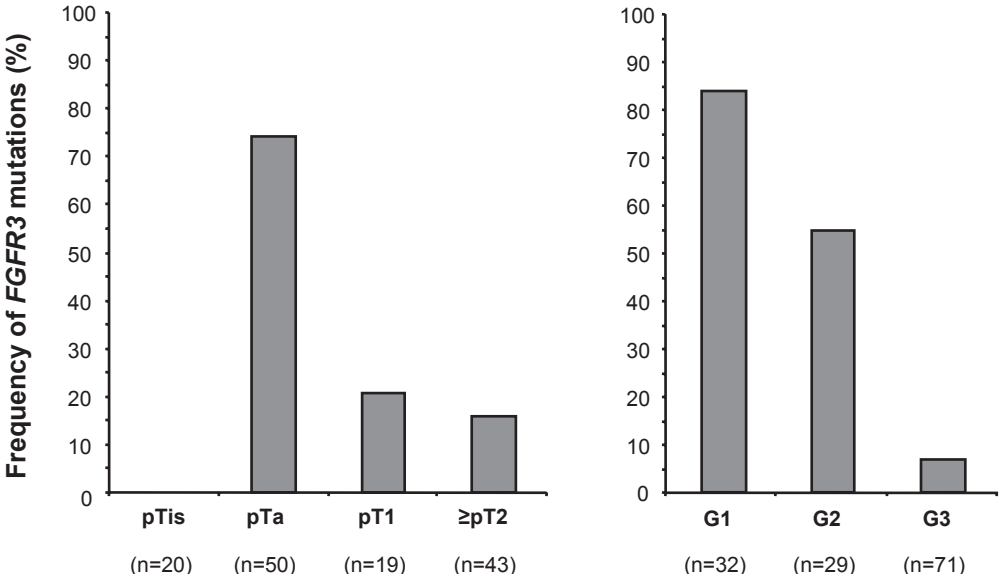
Codon and mutated nucleotide (nt position) are numbered according to cDNA open reading frame corresponding to the FGFR3b isoform. This isoform is produced in epithelial cells and contains two more amino-acids than the FGFR3c isoform present in bones. The codon number according to FGFR3c cDNA open reading frame is indicated in parentheses.

These mutations affected codons 248, 249, 372, 375, 652 (FGFR3b isoform numbering). Four of these 5 mutations were located in the extracellular domain (codons 248 and 249) or transmembrane domain (codons 372 and 375), with the mutated codon encoding a cysteine residue in each case. The fifth mutation, located in the kinase domain (codon 652), resulted in the replacement of a positively charged residue by a negatively charged residue. The S249C mutation

was the most frequent and was found in 33 of the 48 mutated tumors (69%). The Y375C mutation is reported here for the first time in bladder tumors. All 5 mutations identified in bladder carcinomas were identical to the germinal activating mutations responsible for thanatophoric dysplasia, a lethal form of dwarfism. As expected, matched constitutional DNA, available in 15 cases with mutations, contained wild type sequences demonstrating the somatic nature of these mutations.

Relationship between *FGFR3* mutation status, tumor stage and histological grade. We assessed frequency of *FGFR3* mutations in the two types of non invasive bladder cancer (CIS and pTa tumors), in lamina propria-invasive (pT1) and muscle-invasive (pT2-4) tumors. The distribution of *FGFR3* mutations as a function of stage is shown on Figure 1 (leftside).

Figure 1



FGFR3 mutations in relation to stage or grade
Left: Percentage of FGFR3 mutations in carcinoma in situ, pTa, pT1 and pT2-4 tumors.
Right: Percentage of mutations as a funtion of tumor grade.

FGFR3 mutations were observed in 37 of 50 pTa tumors (74%), whereas no mutation was detected in the 20 CIS examined. This difference in mutation frequency between these two groups of tumors was highly significant (P<0.0001; two-sided P, Fisher's exact test). *FGFR3* mutations were detected in 4 of 19 (21%) pT1 tumors and in 7 of 43 (16%) pT2-4 tumors. This difference in mutation frequency of *FGFR3* mutations between pTa and pT1 tumors was highly significant (P<0.0001). The difference in frequency of *FGFR3* mutations between CIS and pT1 was at the limit of significance (P=0.05). In contrast, there was clearly no difference in the frequency of *FGFR3* mutations between pT1 and pT2-4 tumors (P=0.7).

The distribution of *FGFR3* mutations according to histological grade is shown in Figure 1 (rightside). *FGFR3* mutations were detected in 27 of 32 (84%) G1 tumors, 16 of 29 (55%) G2 tumors but in only 5 of 71 (7%) G3 tumors. This association between *FGFR3* mutations and low grade was highly significant (*P*<0.0001; two-sided *P*, Chi-squared test). Table 2 shows the distribution of *FGFR3* mutations as a function of grade for the three groups of tumors classified according to stage (pTa, pT1 and ≥pT2 tumors). A highly significant correlation was found between *FGFR3* mutations and low grade (G1+G2 *versus* G3) within the pTa tumor group (*P*=0.009; two-sided *P*, Fisher's exact test) and within the ≥pT2 tumor group (*P*=0.02; two-sided *P*, Fisher's exact test).The correlation between *FGFR3* mutations and low grade was not significant for pT1 tumors, probably due to the small number of samples in this group.

Table 2
FGFR3 mutations in relation to grade in pTa, pT1 and pT2-4 tumors

Stage	Grade	*FGFR3* wild-type	*FGFR3* mutant	*P*-value*
	G1	5	27 (84%)	
pTa	G2	4	9 (69%)	0.013
	G3	4	1 (20%)	
	G1	0	0	
pT1	G2	1	2 (67%)	0.097
	G3	14	2 (12.5%)	
	G1	0	0	
≥pT2	G2	8	5 (38%)	0.019
	G3	28	2 (7%)	

For each grade, the percentage of tumors with mutations in FGFR3 is indicated.
** Comparison G1+G2 versus G3, two-sided P, Fisher's exact test.*
*** According to the consensus classification of 1998,[21] the pTaG1 + pTaG2 group of tumors corresponds to papillary neoplasms of low malignant potential + low-grade papillary carcinomas, wheras pTaG3 tumors correspond to high-grade pailllary carcinomas.*

DISCUSSION

Germinal point mutations resulting in FGFR3 activation are responsible for dwarfism syndromes. Surprisingly, similar *FGFR3* mutations have also been implicated in tumorigenesis. Somatic *FGFR3* mutations identical to those found in thanatophoric dysplasia (neonatal lethal dwarfism syndrome) and SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans) have been associated with rare cases of human multiple myeloma.[12,13] We recently identified, in a series of 26 bladder and 12 cervix carcinomas, several *FGFR3*-activating mutations previously identified as associated with thanatophoric dysplasia.[8] To investigate futher the role of of *FGFR3* mutations in bladder carcinogenesis, we assessed the incidence of *FGFR3* mutations in a large series of 132 bladder tumors of various stages and grades.

The high frequency of mutations that we found (48 of 132) confirmed that *FGFR3* mutations are a frequent event in bladder carcinomas. Five different

mutations were identified, four of which (R248C, S249C, G372C, K652E) were detected in our previous series: the remaining mutation (Y375C) was identified for the first time in this series. This fifth mutation is also known to be involved in thanatophoric dysplasia. The S249C mutation (TCC → TGC) is the most frequent *FGFR3* mutation in bladder tumors (33 of 48, 69%). In contrast, this mutation was found in only 7 of 62 (11.5%) thanatophoric dysplasia cases, the most common mutation in this syndrome being R248C (CGC → TGC) (26 of 62; 42%; J. Bonaventure, unpublished data). This probably reflects differences in etiology: many *FGFR3* mutations in bladder cancer could be caused by carcinogens whereas the germinal mutations in thanatophoric dysplasia are spontaneous mutations that preferentially create C-to-T transition of CG dinucleotide.

We investigated the relationship between *FGFR3* mutations and tumor stage and found that the frequency of *FGFR3* mutations was very high in pTa tumors (74%). *FGFR3* mutations were present in only 21% of pT1 and 16% of pT2-4 tumors. No mutations were found in the 20 CIS examined. We performed laser microdissection on all of the CIS samples to exclude the possibility of contamination by normal DNA. *FGFR3* is therefore the first gene found to be preferentially mutated in pTa tumors. Unlike *FGFR3* mutations, loss of chromosome 9, the only other frequent genetic alteration found in pTa tumors, is also common in invasive tumors. *FGFR3* mutations were also strongly associated with low grade. No mutations were found in CIS, which were all grade 3 lesions. The percentage of *FGFR3* mutations in non-CIS G3 tumors was low in all stages examined, including Ta and T1 tumors.

Clinical evidence and molecular studies have suggested that there are two different pathways of bladder carcinogenesis, generating two different non-invasive bladder tumors: CIS, which often progress to pT1 and pT2-4 tumors, and pTa, which rarely progress. The highly significant difference in the frequency of *FGFR3* mutations between pTa tumors and CIS ($P<0.0001$) provides additional evidence that these two noninvasive bladder cancers are different entities. Few studies have carried out a genetic analysis of CIS.[2,7] These studies have shown that CIS and invasive tumors have many genetic alterations in common, such as a high frequency of *p53* mutations and specifical chromosomal losses not found in pTa tumors. The high frequency of *FGFR3* mutations in pTa tumors, their absence in CIS and their low frequency in pT1 and pT2-4 tumors are consistent with the model of bladder tumor progression in which the most common precursor of invasive tumors is CIS (CIS → pT1 → pT2-4) and not pTa tumors.[1-6] The mutated invasive tumors may arise from mutated pTa tumors that progress or from CIS that acquire *FGFR3* mutation during progression to invasive tumors. There is also a third possibility that cannot be excluded: some mutated CIS may be the precursors of some mutated pT1 lesions. Studies on larger series and further detailed analyses of the genetic profiles (chromosomal losses and amplifications and *P53* mutations) of recurrent tumors from patients

with progressive disease may help to distinguish between these three possibilities.

Somatic *FGFR3* mutations are probably tumor-specific, as no *FGFR3* mutations were found in 9 random urethelial biopsy samples of normal appearance from 9 patients known to have a *FGFR3* mutation in their papillary lesions (data not shown). The high frequency of *FGFR3* mutations in pTa tumors indicates that FGFR3 activation is a key event in the development of these tumors. It would be interesting to analyze other molecules of the FGFR3 pathway such as FGFR3 ligands or downstream signaling molecules, in pTa tumors without mutations in *FGFR3*.

pTa tumors are the most common type of primary bladder tumor. These tumors rarely progress but recur in more than 50% of cases. Because most of these tumors carry *FGFR3* mutation, the detection of such mutations in urine may provide an accurate additional means of follow-up and identification of tumor recurrences. This could be especially useful for low grade lesions, which are difficult to detect by urine cytology and which harbor *FGFR3* mutations in more than 80% of cases.

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Molecular Grading of Urothelial Cell Carcinoma with *FGFR3* and MIB-1 is Superior to Pathological Grade for the Prediction of Clinical Outcome

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ABSTRACT

Fibroblast growth factor receptor 3 (FGFR3) mutations were recently found at a high frequency in well-differentiated urothelial cell carcinoma (UCC). We investigated the relationship between *FGFR3* status and three molecular markers (MIB-1, P53 and $P27^{kip1}$) associated with worse prognosis and determined the reproducibility of pathological grade and molecular variables. In this multi-center study, we included 286 patients with primary (first diagnosis) UCC. The histological slides were reviewed. *FGFR3* status was examined by PCR-SSCP and sequencing. Expression levels of MIB-1, P53 and $P27^{kip1}$ were determined by immuno-histochemistry. Mean follow-up was 5.5 years (range, 0.4–18.4 years). *FGFR3* mutations were detected in 172/286 (60%) of UCCs. G1-tumors had a *FGFR3* mutation in 88% of cases, G3-tumors in 16%. Conversely, aberrant expression patterns of MIB-1, P53 and $P27^{kip1}$ were seen in 5%, 2% and 3% of G1-tumors and in 85%, 60% and 56% of G3-tumors, respectively. In multivariate analysis with recurrence rate, progression and disease specific survival as endpoints, the combination of *FGFR3* and MIB-1 proved of independent significance in all 3 cases. Using these 2 molecular markers, three molecular grades (mG) could be identified: mG1 (mutation; normal expression), favorable prognosis; mG2 (two remaining combinations), intermediate prognosis; mG3 (no mutation; high expression), poor prognosis. The molecular variables were more reproducible than pathological grade (85–100% vs. 47–61%). The *FGFR3* mutation represents the favorable molecular pathway of UCC. Molecular grading provides a new, simple and highly reproducible tool for clinical decision making in UCC patients.

INTRODUCTION

Urothelial cell carcinoma (UCC) of the urinary bladder is a serious health problem. Over 260.000 cases are diagnosed worldwide each year, accounting for 3.2% of all new cancer cases.[1] For 2001, 54.000 new cases and 12.000 deaths predicted for the USA make UCC the 5th most common cancer and the 9th leading cause of cancer death.[2] The patients can be divided into two groups for clinical management depending on the pathological stage. In most patients, UCC is superficial (i.e. pTa-pT1) at first presentation. After trans-urethral resection, these patients are intensively monitored by cystoscopy since up to 70% experiences one or more recurrences and 10-15% will progress to invasive, potentially lethal UCC.[3-5] Patients with intermediate or high risk superficial UCC usually receive adjuvant intravesical instillations with, in most cases, bacillus Calmette Guérin (BCG).[6,7] Cystectomy, radiation- or systemic chemotherapy are the preferred treatment options in case of invasive (i.e. ≥pT2) disease depending on presence of metastasis and patients co-morbidity and life expectation.

Clinical and pathological variables for prediction of patient's prognosis have been studied extensively.[3,5,8] Although variability in pathological assessment is a recognized problem,[9,10] it still is one of the best predictors of

prognosis. Research efforts of the last decades resulted in a long list of molecular investigations for a possible better assessment of UCC prognosis. Oncogenes, tumor suppressor genes & associated cell cycle proteins, proliferation antigens, growth factors, cell adhesion molecules and chromosomal alterations have been identified in UCC.[11-21] Of these, the *P53* gene is the most investigated molecular marker.[12] Some molecular markers hold considerable promise to assess aggressive UCC behavior (examples: P53, RB, $P27^{kip1}$, P21, Ki-67/MIB-1, E-Cadherin). Only few studies have analyzed the performance of multiple molecular markers. Analogous to clinical and pathological indices, combinations lead to a more tailored prediction of prognosis.[3,8,11,16,18] Nevertheless, the value of molecular markers over clinico-pathological indices is still being questioned and their clinical use limited.[12-15]

Point mutations in the human *FGFR3* gene are well documented in inherited skeletal anomalies associated with dwarfism in most cases, such as achondroplasia and thanatophoric dysplasia.[22-24] In addition, an oncogenic role has been proposed for mutant *FGFR3*.[25,26] Recently, *FGFR3* mutations were found in over 40% of UCC cases[27-29] whereas a low frequency was observed in multiple myeloma[30] and cervical carcinoma[31]. Surprisingly, *FGFR3* mutations in UCC were related to favorable disease with 84% of pTaG1 tumors having a mutation as compared to 7% of ≥pT2G3 tumors.[29] Moreover, in a prospective study with a 1-year follow up, we showed that *FGFR3* mutations were associated with a low recurrence rate of superficial UCC.[28] However, so far, nothing is known on the *FGFR3* mutation in relation to long term clinical follow up or other molecular markers.

Based on these initial observations, we designed this multi-center study to compare the relationship between three immuno-histochemical markers associated with worse UCC prognosis i.e. the proliferation marker MIB-1, P53 nuclear overexpression and reduced expression of the cell cycle inhibitor $P27^{kip1}$ and the *FGFR3* mutation in a large patient group. In addition, we investigated the reproducibility of pathological grading and molecular variables in the same series.

PATIENTS AND METHODS

Patients. This study consists of 286 patients with papillary UCC. They were between 23 and 90 years old (mean, 65.7±12.0 years). None of them had a hereditary skeletal disorder documented. The patient and tumor characteristics at first diagnosis are reported in Table1.[32-34] Of the 286 patients, 246 (86%) had superficial (i.e. pTa, pT1) UCC. The patients were seen at the Urology departments of the University Hospital Rotterdam (N=139), the Sint Franciscus Gasthuis (N=121) or at one of 3 Urology departments in the Rotterdam surroundings (N=26). A paraffin-embedded, formalin fixed tissue block was obtained. These were freshly cut into 4 μm thick sections and mounted on amino alkylsilane (AAS)-coated glass slides for immuno-histochemistry. Standard H&E slides were made from the last cut section. These slides were reviewed by a

specialized genitourinary pathologist (TvdK) with the criteria of the 1998 WHO/ISUP classification system.[34] Furthermore, two additional pathologists with a special interest in uro-pathology (AJ, EO) graded 210 slides for reproducibility purposes.

Table 1
The patient and tumor characteristics of the 286 studied cases.

Variable		Patients	
		No.	%
sex	Female	69	24
	Male	217	76
Multiplicity	Solitary	212	74
	Multiple	74	26
Site	Upper UT	21	7.5
	Lower UT	265	92.5
Localization	Bladder neck	13	4.5
	Anterior wall	14	5.0
	Dome	10	3.5
	Posterior wall	23	8.0
	Trigone/ostia	37	13
	Left lateral wall	44	15
	Right lateral wall	46	16
	Ureter	7	2.5
	Pyelum	14	5.0
	Multiple	65	23
	Unknown	13	4.5
Tumor size (diameter)	< 1 cm	33	11
	1-3 cm	122	43
	3-5 cm	66	23
	> 5 cm	65	23
Stage (TNM '97)+	pTa	183	64
	pT1	63	22
	pT2	32	11
	pT3	6	2.0
	pT4	2	1.0
Grade (WHO '73)*	G1	93	32
	G2	125	44
	G3	68	24
Grade (WHO/ ISUP '98)^	PUN-LMP	84	29
	LG-PUC	94	33
	HG-PUC	108	38
Associated CIS	Yes	19	6.5
	No	267	93.5
Total		286	100

*+Stage (TNM '97): according to TNM 1997 guidelines.[32] *Grade (WHO '73): Routine morphological grading according to the 1973 WHO classification system.[33] ^Grade (WHO/ISUP '98): Reviewed morphological grading by one pathologist according to the 1998 WHO/ISUP classification system.[34]*

Abbreviations: No Pts, number of patients; UT, urinary tract; PUN-LMP, papillary urothelial neoplasia of low malignant potential; LG-PUC, low grade – papillary urothelial carcinoma; HG-PUC, high grade – papillary urothelial carcinoma; CIS, carcinoma in situ.

Fifty UCCs from the archive served as a teaching set to agree upon grading parameters.[34] 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.

Follow up. Clinical information and follow up data were collected by chart review. Disease specific survival was determined for all patients. For patients with primary superficial UCC, recurrence, recurrence rate and progression were additional endpoints. Recurrence rate was defined as the number of histologically proven recurrences divided by years of follow up. Hence, the recurrence rate takes into account the clinical course of patients during a longer interval and not only covers the time to first recurrence. Progression was defined as development of invasive (i.e. ≥pT2) UCC. Mean follow up was 5.5 years (range, 0.4-18.4 years) for all patients and 5.9 years (range, 0.8-18.4 years) for the patients with superficial UCC. After superficial UCC, 153 (62%) patients experienced one or more recurrence(s). Median time to first recurrence was 1.0 years (range, 0.2-9.7 years). An upper-tract (ureter, renal pelvis) recurrence was found in 15 (5.2%) patients. Sixty patients had a recurrence rate of more than 0.50 per year. Progression was found in 28 (11.4%) patients. Median time to progression was 3.1 years (range, 0.3-14.3 years). Twenty-six (9.1%) patients died of UCC. Fifteen of these were initially diagnosed with superficial UCC. Median time to decease was 3.8 years (range, 0.4-14.2 years). During follow up, 160 patients received intravesical treatment involving BCG in 101 cases. A cystectomy was performed in 22 patients. Eleven of these were performed after progressive disease. Twenty-nine patients of whom 22 with curative intent were treated by radiotherapy and 14 patients received systemic chemotherapy for metastatic UCC. The patients were censored at their last visit to the Urology department or at the date of their decease.

Immuno-histochemistry. All cases were routinely processed with 3 monoclonal antibodies by the immuno-histochemical laboratory of the University Hospital Rotterdam as described.[35] The incubation with the primary antibody P53 (clone DO-7, DAKO, Denmark) took 30 minutes at an optimal dilution of 1:200 in PBS/BSA 5% or overnight at 4°C with $p27^{kip1}$ (clone 1B4, Novocastra, Newcastle, UK) at 1:40. The murine antibody MIB-1 (Immunotech, Marseille, France) against the Ki-67 antigen was incubated for 30 minutes at 1:200. Positive and negative controls were always included. The conventional avidin-biotin complex method was applied for all immuno-stainings as described.[35] Two medical researchers (BvR, AV) independently assessed the slides without knowledge of clinical data. The slides were scored in a semi-quantitative manner, estimating the percentage of tumor cells that show positive nuclear staining. It is assumed that aberrant expression of MIB-1, P53 and $P27^{kip1}$ are associated with worse patient

prognosis. Therefore, in case of heterogeneity, the parts within the tumor that showed highest (MIB-1, P53) or lowest ($P27^{kip1}$) positive to total ratio were particularly assessed. This was performed if these regions comprised at least 10% of the tumor load in the examined tissue section. Cutoff levels were defined at 25% (MIB-1), 10% (P53) and 50% ($P27^{kip1}$) using a teaching set of 40 UCCs collected from a previous study[28]. In case of discrepancy between the observers, the slides were reassessed in a combined session without the information of the previous scores.

***FGFR3*-mutation analysis.** The H&E slides made from the last cut section were used as templates for the microdissection procedure. A representative area of the tumor was dissected avoiding contamination with stroma, normal mucosa or leukocytes. The samples used for *FGFR3* mutation analysis contained a minimum of 70% tumor cells, as assessed by histological examination. The DNA was extracted using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). The *FGFR3* mutation analysis was performed by polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis as described.[26,28,29] In brief, four regions encompassing all activating *FGFR3* mutations previously described in severe skeletal dysplasias and cancers were amplified. The primer-sequences were as reported.[29] The ^{32}P-labeled PCR-products were separated on 6% polyacrylamide gels in 0.2x (exon 7) or 1x SSCP-buffer (exons 10, 15 & 19) (10x SSCP-buffer = 0.5M Tris-borate, 1mM EDTA). Samples with a shift were sequenced with T7 Sequenase v2.0 (Amersham life Science, Inc., Cleveland, OH). In addition, DNA extracted from venous blood for control purposes was available from 75 patients who attended the Urology department of the University Hospital Rotterdam. Five of these samples were used as negative controls for sequencing. When we detected a new *FGFR3* mutation in UCC and no blood-DNA was available, DNA of paraffin-embedded, non-malignant tissue of the same patient was isolated and processed as described above to examine whether the mutation was somatic. These laboratory analyses were performed without knowledge of clinical data. To test the reproducibility of the *FGFR3* mutation analysis, eighty-one DNA samples were analyzed in two institutes (Josephine Nefkens Inst., Rotterdam and Inst. Curie, Paris).

Statistical analysis. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for the data documentation and analysis. Clinical and pathological variables were combined in the Clinico-pathological index constituted of 4 adverse tumor characteristics in primary superficial UCC.[8] The significance of single and combinations of variables with regard to follow up was analyzed by applying the Kaplan-Meier method. ANOVA (for comparison of means) was used for comparison of recurrence rate. Two-sided Fisher's exact test was applied for the relation of *FGFR3* mutations in papillary UCC and associated carcinoma in situ (CIS). Multivariate Cox regression analysis was used to find independent prognostic

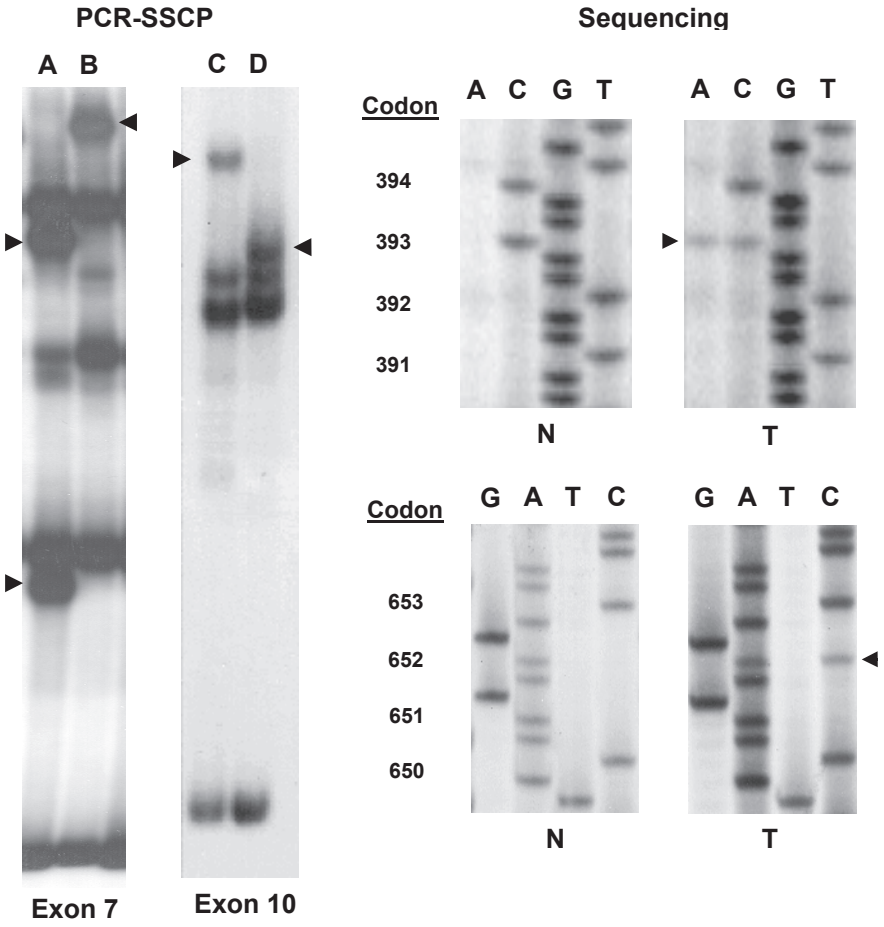
factors. The odds ratios and their 95% confidence intervals were calculated for the significant predictors in the multivariate analyses. Statistical significance was assumed if $P<0.05$.

RESULTS

We studied the performance of four molecular markers in a series of 286 primary UCC.

***FGFR3* mutations.** We detected activating *FGFR3* mutations in 172 (60%) of the 286 tumors. In the 246 superficial UCCs, 164 (67%) tumors had a mutation in the *FGFR3* gene. Examples of these mutations determined by PCR-SSCP and sequencing are shown in Figure 1.

Figure 1



FGFR3 mutations. PCR-SSCP: Arrowheads indicate aberrant bands with the mutations R248C (A), S249C (B), G372C (C) and Y375C (D). Sequencing: The C to A transition at codon 393 results in A393E and the A to C transition at codon 652 results in K652T. The mutations are absent in normal DNA. Abbreviations: N, normal DNA; T, tumor (UCC) DNA.

The mutations were found in 6 different codons (Table 2). In 3 cases, two concurrent mutations were found in one tumor. S249C accounted for 120 (69%)

of the 175 mutations. We found no difference between S249C and other *FGFR3* mutations in relation to clinical data (not shown). No activating *FGFR3* mutations were found in 75 matched normal (blood) DNA samples nor in normal tissue confirming the somatic nature of *FGFR3* mutations in UCC.

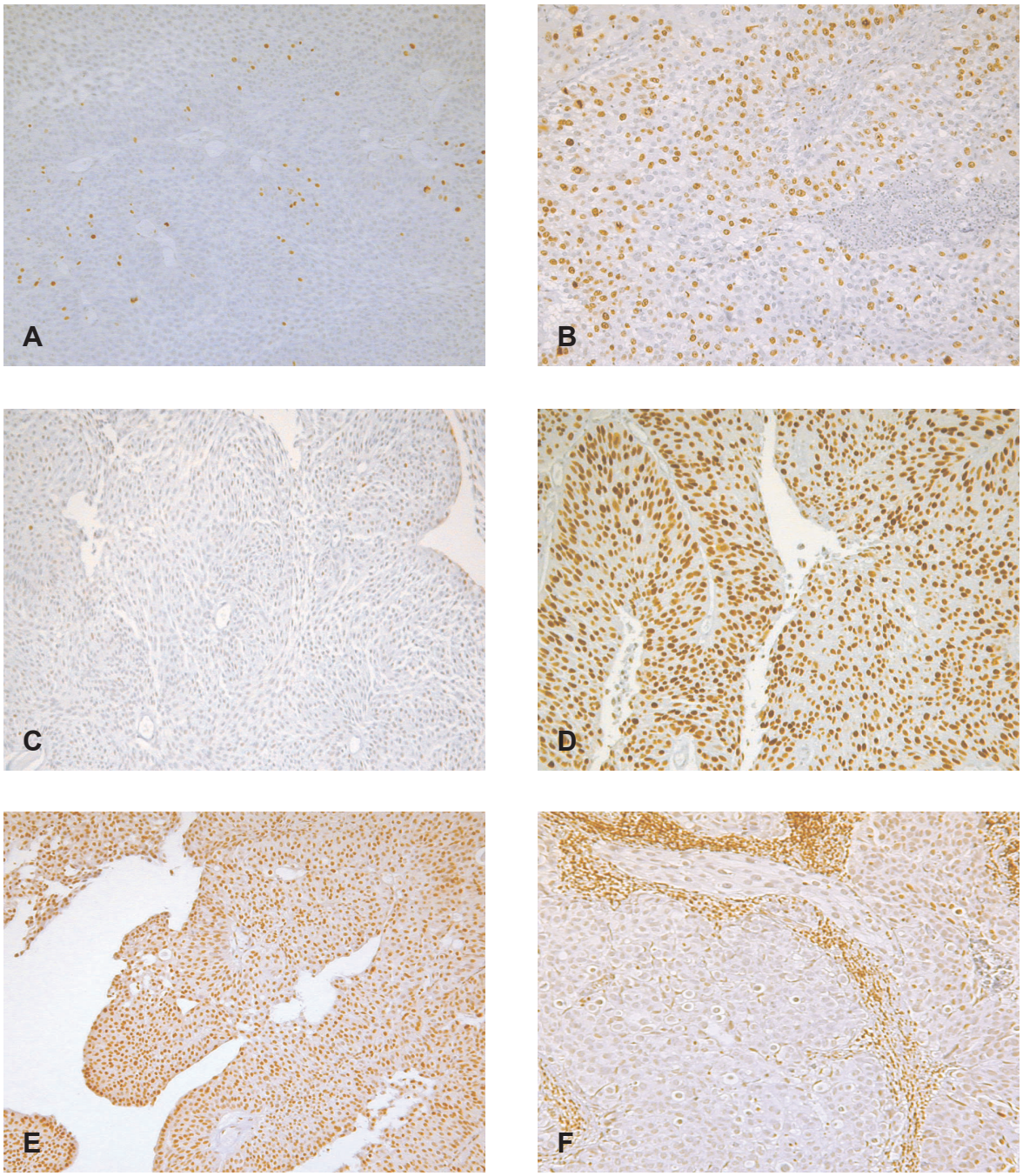
Table 2

Somatic mutations of the FGFR3 gene in the studied population.
The codon and nucleotide (nt) numbering refers to the open reading frame of the FGFR3b isoform that is expressed in epithelial cells.[26] Three cases showed two concurrent FGFR3 mutations in one tumor. No activating FGFR3 mutations were found in exon 19.

Exon(s)	Activating *FGFR3* mutations Mutation, predicted effect	Number of tumors
7	nt742 C→T, R248C	12
7	nt742 ins GCT, R248RC	1
7/7	nt742 C→T, R248C and nt746 C→G, S249C	1
7	nt746 C→G, S249C	117
7/10	nt746 C→G, S249C and nt1114 G→T, G372C	1
7/10	nt746 C→G, S249C and nt1178 C→A, A393E	1
10	nt1114 G→T, G372C	7
10	nt1124 A→G, Y375C	25
10	nt1178 C→A, A393E	3
15	nt1954 A→G, K652E	1
15	nt1955 A→C, K652T	1
15	nt1955 A→T, K652M	2

Molecular markers and histo-pathology. Examples of normal and aberrant expression of the 3 immuno-histochemical markers are shown in Figure 2. Table 3 shows the correlation of the molecular markers and histo-pathological data. The highest percentage (88%) of *FGFR3* mutations was seen in G1-tumors, the lowest percentage (16%) in G3-tumors. Conversely, aberrant expression patterns of MIB-1, P53 and $P27^{kip1}$ were seen in 5%, 2% and 3% of G1-tumors and in 85%, 60% and 56% of G3-tumors, respectively. Therefore, the *FGFR3* mutation is generally associated with a favorable histo-pathological diagnosis, whereas aberrant expression of MIB-1, P53 and $P27^{kip1}$ point to unfavorable disease. When we compared the performance of the molecular markers in the 22 patients who underwent cystectomy, we retrospectively observed aberrant MIB-1 expression in all 22 patients whereas a *FGFR3* mutation was only observed in 3 of these patients. Combining two UCC related molecular features, i.e. *FGFR3* mutation and high MIB-1 expression, we could define 249 (87%) tumors. This combination defined the highest percentage of UCC.

Figure 2



Immuno-histochemical staining showing normal (A, C, E) and aberrant (B, D, F) protein expression levels. The molecular markers used in this study are the proliferation marker MIB-1 (A, B), P53 (C, D) and the cell cycle inhibitor $P27^{kip1}$ (E, F). Example F shows that stromal cells may serve as internal control for $P27^{kip1}$ staining.

Table 3

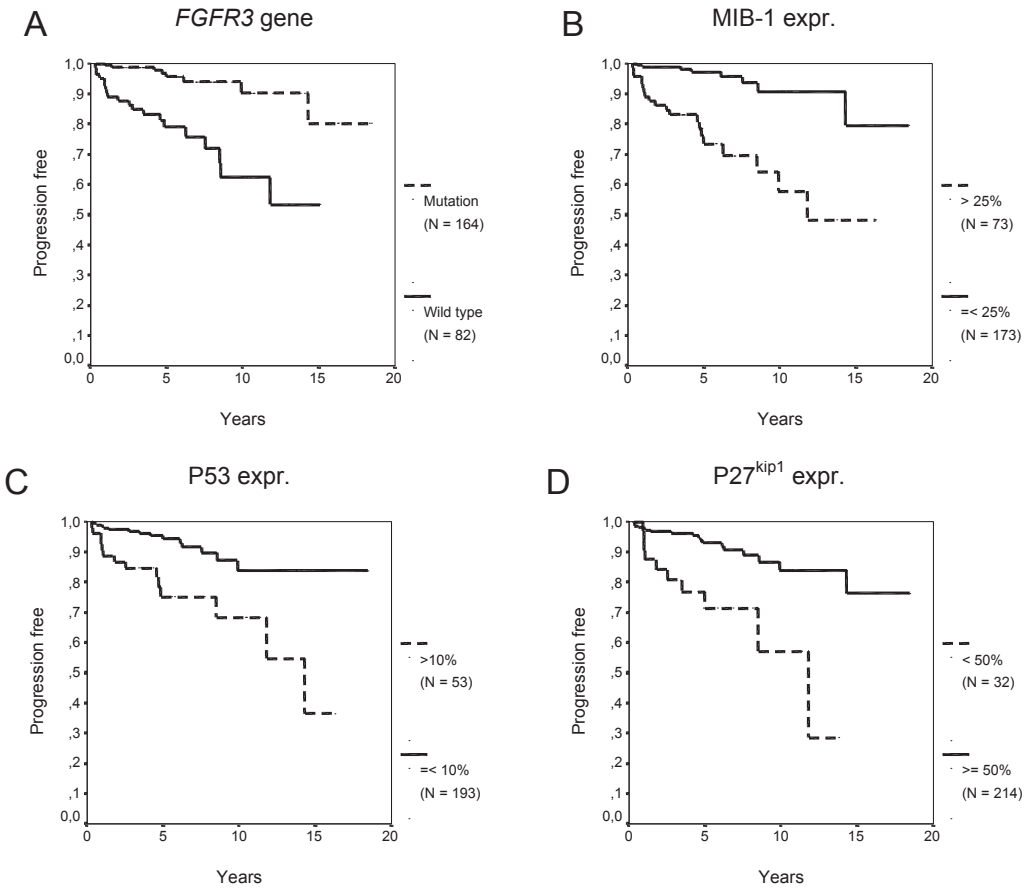
Comparison of the four molecular markers and histo-pathology.
For each molecular marker, its relation to Grade in pTa, pT1 and invasive (≥pT2) urothelial cell carcinoma is shown. The % of positive, UCC-related molecular features (FGFR3 mutation, MIB-1>25%, P53>10% and P27^{kip1}<50%) are given for each histo-pathological category between parentheses. The WHO 1973 classification system[33] was used as it allows a better comparison with previous studies and because we observed no substantial difference for the two morphological grading systems[33,34] concerning the molecular features in this series.

		FGFR3 gene		MIB-1 expr.		P53 expr.		P27^{kip1} expr.		Total
		wt	mt (%)	≤25 %	>25% (%)	≤10 %	>10% (%)	≥50 %	<50% (%)	
pTa	G1	11	81 (88)	87	5 (5)	90	2 (2)	89	3 (3)	92
	G2	24	59 (71)	62	21 (25)	68	15 (18)	75	8 (10)	83
	G3	7	1 (13)	0	8 (100)	2	6 (75)	5	3 (38)	8
pT1	G1	0	1 (100)	1	0 (0)	1	0 (0)	1	0 (0)	1
	G2	18	17 (49)	16	19 (54)	21	14 (40)	27	8 (23)	35
	G3	22	5 (19)	7	20 (74)	11	16 (59)	17	10 (37)	27
≥pT2	G2	4	3 (43)	2	5 (71)	4	3 (43)	4	3 (43)	7
	G3	28	5 (15)	3	30 (91)	14	19 (58)	8	25 (76)	33
Total		114	172 (60)	178	108 (38)	211	75 (26)	226	60 (21)	286

wt: wild type (no mutation), mt: mutation, expr.: expression

A molecular grading model for prediction of prognosis. The first endpoint of interest was the prediction of disease progression for patients with superficial UCC. The corresponding Kaplan-Meier analyses for the performance of the four molecular markers are shown in Figure 3A-D. This figure illustrates, analogous to the histo-pathological data in Table 3, that UCC patients with a *FGFR3* mutation have a significantly better prognosis than those without the *FGFR3* mutation and that the opposite is the matter in patients with aberrant expression of an immuno-histochemical marker. These results prompted us to perform multivariate analysis for the prediction of progression with the single and combinations of variables listed in Table 4. The combination *FGFR3*/MIB-1 ($P<0.001$) and the clinico-pathological index ($P=0.007$) proved independent predictors of progression. Further details on multivariate analyses are explained below.

Figure 3



The Kaplan-Meier analyses of the 4 molecular markers for disease progression. ***A)*** *FGFR3 gene,* ***B)*** *MIB-1 expression,* ***C)*** *P53 expression and* ***D)*** *P27^{kip1} expression. P-values (log-rank) were <0.001 for each marker. The dashed lines show the follow up of patients with positive, UCC-related molecular features. Abbreviations, see footnote Table 3.*

Figure 4A shows the Kaplan-Meier plot for the combination *FGFR3*/MIB-1. The difference in behavior between the groups *FGFR3* mutation / MIB-1 normal (N=138) and *FGFR3* wild type / MIB-1 high (N=47) was evident. Interestingly, the 61 remaining patients with tumors characterized by no *FGFR3* mutation and normal MIB-1 expression or by a *FGFR3* mutation and high MIB-1 expression behaved more or less the same representing an intermediate prognosis with regard to disease progression. These results from Figure 4A led us to propose a molecular grading model for UCC distinguishing three molecular grades (mG1-3): mG1 (favorable prognosis), mG2 (intermediate prognosis) and mG3 (poor prognosis) as shown in Figure 4B. This Figure illustrates the different disease courses for the various molecular Grades. The combinations of *FGFR3* status with P53 and P27^{kip1} expression were only significant predictors in univariate analysis (not shown).

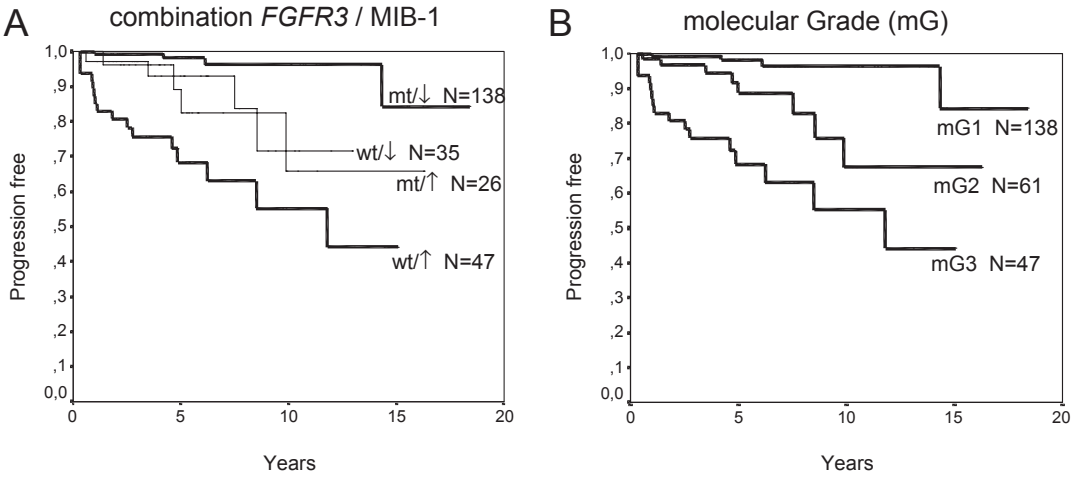
Table 4

Variables for multivariate analysis to predict patient's prognosis.

Single variables:	Combination of variables:
Age	Clinico-pathological index^
Hospital	*FGFR3*/MIB-1
Sex	*FGFR3*/P53
Multiplicity	*FGFR3*/$P27^{kip1}$
Site	MIB-1/P53
Localization	MIB-1/$P27^{kip1}$
Tumor size	P53/$P27^{kip1}$
Stage (TNM '97)*	
Grade (WHO '73)*	
Grade (WHO/ISUP '98)*	
Associated CIS	
FGFR3 status	
MIB-1	
P53	
$P27^{kip1}$	

**See fotnote Table 1. ^Index adopted from Allard et al.[8] based on 4 adverse tumor characteristics (ATC): i.e. tumor-size >3cm, Stage pT1, Grade G2-3 and multifocality.*

Figure 4



Molecular grading of UCC. ***A)*** *Kaplan-Meier plot for progression-free survival based on FGFR3/MIB-1 status, i.e. mt (mutant) or wt (wild type) FGFR3 and ↑ (high) or ↓ (low) MIB-1 staining. Note that the combinations mt/↓ and wt/↑ behave similarly.* ***B)*** *Molecular Grading based on these parameters: mG1: mt FGFR3 / ↓ MIB-1; mG2: mt FGFR3 / ↑ MIB-1 and wt FGFR3 / ↓ MIB-1; mG3: wt FGFR3 / ↑ MIB-1.*

Multivariate analyses illustrate the value of molecular Grade. We also performed multivariate analyses for the other study endpoints, i.e. recurrence, recurrence rate and disease specific survival. Table 5A shows the significant predictors in multivariate analysis for patients with superficial UCC. Besides progression, the molecular Grade was also an independent predictor for recurrence rate and disease specific survival. The recurrence rate per year

calculated for the independent variables, i.e. molecular- and morphological Grade is shown in Figure 5. In Table 5B, the significant predictors in multivariate analysis for all patients (N=286) concerning disease specific survival are listed.

Table 5A
Independent predictors in superficial urothelial cell carcinoma (N=246).

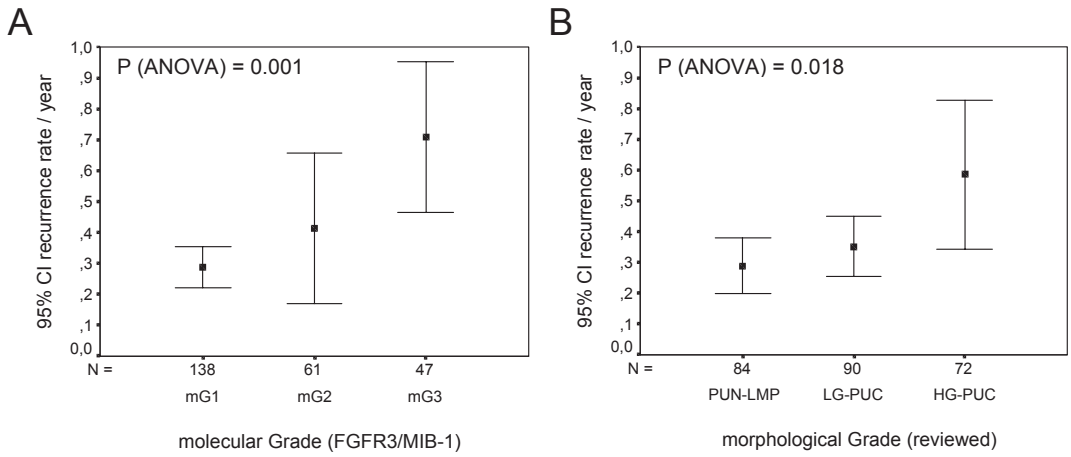
Prognostic Factor		Odds Ratio	95% CI	P-value
Recurrence				
Multiplicity	Solitary	1		
	Multiple	1.8	1.3 – 2.5	0.002
Recurrence rate (>0.50 / year)				
Grade 1998*	PUN-LMP	1		
	LG-PUC	2.6	1.2 – 5.7	
	HG-PUC	2.9	1.2 – 6.9	0.019
FGFR3/MIB-1**	mG1	1		
	mG2	1.2	0.6 – 2.2	
	mG3	2.5	1.2 – 5.4	0.046
Progression				
Clinico-pathological index^:	0-1 ATC	1		
	2 ATC	3.8	0.5 – 32.3	
	3-4 ATC	4.8	0.6 – 39.7	0.007
FGFR3/MIB-1**	mG1	1		
	mG2	4.5	1.3 – 16.0	
	mG3	9.3	2.9 – 29.8	<0.001
Disease specific survival				
FGFR3/MIB-1**	mG1	1		
	mG2	15.6	1.7 – 149.2	
	mG3	19.6	1.4 – 273.8	0.008

Table 5B
Independent predictors in urothelial cell carcinoma for disease specific survival (N=286)

Prognostic Factor		Odds Ratio	95% CI	P-value
Disease specific survival				
Stage	pTa	1		
	pT1	1.5	0.5 – 4.4	
	≥pT2	8.2	2.7 – 24.9	<0.001
FGFR3/MIB-1**	mG1	1		
	mG2	8.5	1.9 – 45.7	
	mG3	10.0	2.0 – 48.5	<0.001

** See footnote Table 1 for explanation & abbreviations*
^ Table 4 for explanation & abbreviation
*** Figure 4 for explanation*
CI: Confidence Interval
mG: molecular Grade

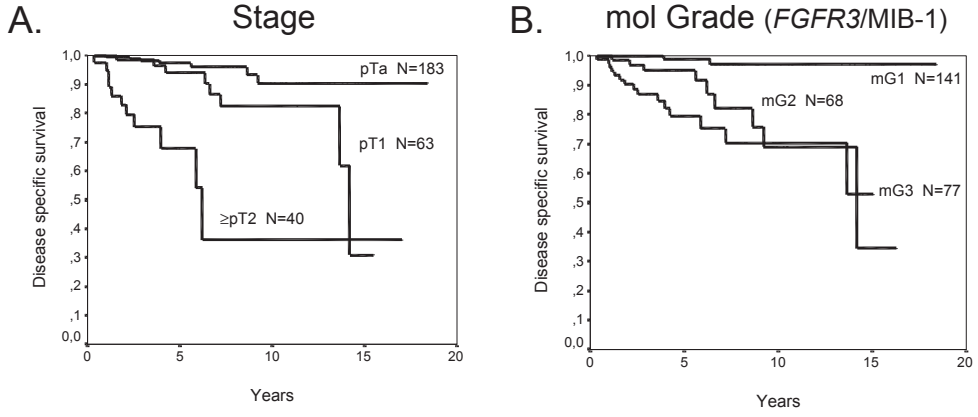
Figure 5



The recurrence rate per year for molecular (A) and morphological (B) grading of UCC. The solid dots indicate the mean recurrence rate per year for each category. Abbreviations, see Table 1 and Figure 4.

Figure 6 shows the Kaplan-Meier plots for the independent predictors of disease specific survival, i.e. pathological Stage and molecular Grade. In addition, we investigated the possible influence of treatment variables, i.e. intravesical treatment for progression and cystectomy for disease specific survival. When we added these covariates to the multivariate analyses, they were not significant.

Figure 6



Independent variables in multivariate analysis for disease free survival (N=286). ***A)*** *pathological Stage* ***B)*** *molecular Grade (FGFR3/MIB-1) were the two independent predictors. P (log-rank) <0.001 for both variables. Abbreviations: mol, molecular; mG, molecular Grade; N, number of patients who entered follow up.*

To further clarify the relation between the molecular grading and disease specific survival as depicted in Table 5B and Figure 6B, we calculated the absolute numbers and time to decease for each molecular Grade. Of the patients with mG1, two patients (1.4%) died of UCC. Nine patients (13%) with mG2 and 15

patients (20%) with mG3 died of UCC. Median time to decease was 5.1, 6.2 and 2.3 years respectively, for mG1, mG2 and mG3. These data pointed to two conclusions. Firstly, the molecular variables were at least as predictive as clinico-pathological indices and secondly, combinations of variables provided a more accurate prediction of prognosis than single variables.

***FGFR3* status and carcinoma in situ.** The presence of CIS next to a papillary UCC generally points to a worse prognosis. Therefore, we analyzed the incidence of *FGFR3* mutations in patients with associated CIS. In the 19 cases with associated CIS, only 5 mutations were found (P=0.003, 2-sided Fisher's exact test). In addition, only 14 mutations were detected in the 42 patients who had associated CIS with their primary UCC or who developed CIS during follow up (P<0.001). Taken together, *FGFR3* mutations were, as expected, not frequent in UCC patients with associated CIS. These data are in line with the absence of *FGFR3* mutations in CIS itself.[29]

Reproducibility. In addition to prognosis, we determined the reproducibility of molecular assays and pathological grading. The histological slides were reviewed by 3 pathologists with a special interest in uro-pathology. Two medical researchers assessed immuno-histochemisty scores. Because the *FGFR3* mutation analysis was reproducible in 100% of cases, the variability of molecular Grade was only dependent on immuno-histochemistry. Table 6 clearly demonstrates the superior reproducibility of the molecular markers over pathological grade.

Table 6
Reproducibility of molecular variables and pathological grade.

Variable	Source of Variability	No. of Patients	% Agreement
MIB-1	2 scorers / 1 slide	286	91
P53	2 scorers / 1 slide	286	88
$P27^{kip1}$	2 scorers / 1 slide	286	85
FGFR3 status	2 institutes / 1 DNA sample	81	100
Pathological Grade	3 pathologists (A,B,C) / 1 slide	210	47 (A vs. B) 60 (A vs. C) 61 (B vs. C)

DISCUSSION

Intense research efforts are being made to identify and characterize molecular markers for various malignancies. However, the value of these markers over traditional clinico-pathological indices for diagnosis and prognosis may still be questioned. We here show that molecular markers can be used to assess patients prognosis for UCC more accurately and reproducibly than with traditional clinico-pathological indices. In this respect, the identification of the *FGFR3* mutation as a selective marker for favorable disease is of major importance.

Multiple endpoints were determined for the prediction of prognosis in our series of patients. Firstly, definitive cure reflected by a long recurrence free follow up is most likely to be achieved in patients with solitary tumors, as reported earlier.[5] The independent predictors for assessment of the biological behavior of superficial UCC reflected by recurrence rate and disease progression were the molecular Grade, the clinico-pathological index and the morphological Grade assessed by a single expert-pathologist. For disease specific survival, a longer follow up may be needed and radical treatment (cystectomy) may give an additional bias. Nevertheless, the molecular Grade (*FGFR3*/MIB-1) again proved to be of independent significance. The data obtained from multivariate analyses indicated that the molecular variables were at least as predictive as clinico-pathological indices and that combinations provided a more accurate prediction of prognosis than single variables.

A surprise to us was the finding that patients with aberrant expression of MIB-1, P53 or $P27^{kip1}$ had a better prognosis when a coexisting *FGFR3* mutation was present in their UCC, i.e. the presence of a *FGFR3* mutation was “protective”. Our finding that these patients had a similar prognosis as patients with no *FGFR3* mutation and normal expression of these proteins made it feasible to combine these groups and to propose a molecular grading model for UCC based on 3 molecular Grades (mG1-3). Because the combination of *FGFR3* and MIB-1 proved, in contrast to other molecular combinations, of independent value for several of our study endpoints in multivariate analyses, we advocate this combination to be used for further validation of the molecular Grade in clinical settings.

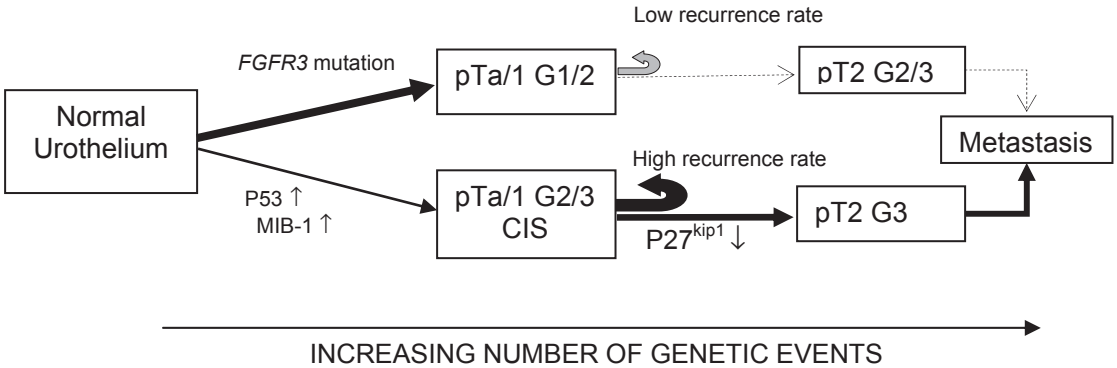
The poor reproducibility of pathological stage and grade is a recognized problem and a major concern for clinicians. Grade assessment may differ in 40-50% of cases.[9,10] Our study confirmed this high inter-observer variability. We also investigated the molecular markers and clearly demonstrated their superior reproducibility. To our knowledge, the present study is the first to compare the reproducibility of pathological grading and molecular variables on the same series of patients. McShane et al. performed an elaborate study, which addressed the reproducibility of P53 immuno-histochemistry.[36] They found a 91% agreement, which is comparable with our result of 88%. Furthermore, inter-observer variability seems not an issue for the *FGFR3* mutation since this analysis appeared highly reproducible with 100% concordance. Moreover, the identification of *FGFR3* mutations was a simple procedure and more sophisticated and faster ways for detection of single nucleotide changes have recently been developed.[37] With our method, the molecular Grade may be provided in the same time frame that is needed for a routine pathological examination. The intermediate Grade 2 tumors are often bothersome for clinical decision making. Therefore, an additional advantage of molecular grade compared to morphological grade was the relative low number of Grade 2 tumors,

i.e. 24% mG2 vs. 44% G2 [33]. In conclusion, molecular grading composed of 3 mGs is more reproducible than pathological assessment.

FGFR3 mutations occur in 60% of primary UCC and their presence was generally linked to a favorable disease course. As expected from earlier studies, aberrant expression of MIB-1, P53 or $P27^{kip1}$ pointed to unfavorable disease characteristics.[11,16-20] By combining the "favorable" *FGFR3* mutation with the "unfavorable" high MIB-1 expression, 87% of UCC could be characterized. Only 12% of these UCC were positive for both markers. This supports a model in which the *FGFR3* mutation and the MIB-1 marker define two pathways of UCC pathogenesis covering almost 90% of the patients (Figure 7). This model for two molecular pathways of UCC resembles the model originally proposed by Spruck et al.[38] However, our model is the first to provide a selective molecular marker for patients with a favorable prognosis. Another important addition to the model of Spruck et al.[38] is our observation that superficial UCCs with a *FGFR3* mutation have a low recurrence rate (reference nr.28 and present study). We included this feature into our model to discern more accurately between the favorable superficial UCCs and the unfavorable ones as this is not evident from histo-pathology alone (Table 3 and Figure 7). Genetic alterations affecting many different chromosomes have been observed in CIS and papillary high-grade UCC.[13,39,40] However, besides *FGFR3* mutations, deletions of chromosome 9 are the only other frequent (>35%) events in low grade and stage papillary UCC, indicating that loss of chromosome 9 occurs early in the development of UCC. Unfortunately however, chromosome 9 deletions are frequently found throughout all UCC stages and grades and, thus, can not be used for prognostic purposes.[38-43]

Figure 7

MOLECULAR PATHWAYS OF UCC



Two-pathway model for disease pathogenesis of urothelial cell carcinoma. Arrow thickness is indicative for the percentage of tumors. Chromosomal alterations, not examined in this study have not been included in the interest of clarity but are represented by the bottom arrow.[11,13,14,21,38-43] Abbreviations: FGFR3, fibroblast growth factor receptor 3 gene; mt, mutation; ↑, elevated expression (MIB-1, P53); ↓, reduced expression ($p27^{kip1}$); CIS, carcinoma in situ.

From the molecular point of view, the *FGFR3* mutation and its apparent positive identification of low risk patients is also very intriguing. It has been shown that mutant *FGFR3* can transform NIH 3T3 cells when targeted to the cell membrane and that these cells are tumorigenic in nude mice.[44,45] Chesi et al. showed that, in multiple myeloma, *FGFR3* and *RAS* mutations are mutually exclusive and both mutations lead to activation of the same downstream pathway (MAPK).[45] Thus, it would be interesting to analyze *RAS* mutations and *FGFR3* mutations in the same UCCs. So far, the frequency of *RAS* mutations in UCC has been controversial.[21] Assuming a link between favorable UCC and *RAS* mutations, some reported studies might have included too many patients with advanced disease.[21] In addition to *RAS* mutations, it would be interesting to analyze other molecules of the FGFR3 signal-transduction pathway.

The identification of the *FGFR3* mutation as the representative of the favorable pathway in this series of 286 primary UCCs is of major importance for patients, clinicians and scientists. Before this mutation and its apparent positive identification of low-risk patients were noted, only molecular indicators of worse prognosis were available in UCC. We proposed a simple, highly reproducible method to determine the molecular Grade of UCC which proved superior to traditional clinico-pathological indices. These results further underline the need for both worse and favorable molecular markers to accurately predict disease course and suggest that this approch will be of value for other types of malignancy as well. Our results warrant the need for future prospective trials and these may confirm the idea that the molecular Grade can be used in clinical decision making with regard to the frequency of cystoscopical follow up, adjuvant intravesical instillations or timing of radical treatments like cystectomy. In addition, the “favorable” *FGFR3* mutation remains a major target for scientists to unravel its mechanisms of action.

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10

Frequent *FGFR3* Mutations in Urothelial Papilloma

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ABSTRACT

Activating point mutations in the *FGFR3* gene occur frequently in low grade and low stage bladder carcinomas whereas they are rare in high-grade carcinomas. This study investigates the incidence of *FGFR3* mutations in 12 urothelial papillomas and 79 pTaG1 tumors which were regraded according to the 1998 WHO/ISUP classification system resulting in 62 papillary urothelial neoplasms of low malignant potential (PUN-LMP) and 17 low grade papillary urothelial carcinomas (LG-PUC). We also performed *FGFR3* mutation analysis of 21 ovarian Brenner tumors. We detected 77 cases with a mutation in the *FGFR3* gene. The mutations were exclusively found in bladder neoplasms. In urothelial papilloma, generally considered a benign lesion, we found 9/12 (75%) mutations. This report is the first to describe a genetic defect in urothelial papilloma. A comparable percentage of mutations was found in PUN-LMP (85%) and LG-PUC (88%). No mutations were found in matched normal DNA from bladder tumor patients. The mean follow up was 5.78 years (range, 0.21-17.60 years). Five patients developed high-grade papillary urothelial carcinoma 2½ to 12 years after first diagnosis. Two patients died of bladder cancer. The mean number of recurrences (recurrence rate) per year was 0.03, 0.21 and 0.46, respectively, for papilloma, PUN-LMP and LG-PUC. Urothelial papilloma is a rare lesion with a benign natural behaviour compared to PUN-LMP and LG-PUC of the bladder. However, from a molecular perspective, papillomas should be classified together with all well differentiated urothelial neoplasms.

INTRODUCTION

The diagnostic criteria for urothelial papilloma are essentially identical in the 1973 WHO classification system and the 1998 WHO/International Society of Urological Pathology (WHO/ISUP) classification system for papillary urothelial neoplasms.[1,2] Urothelial papilloma is generally considered a separate diagnostic entity with a benign natural behaviour by most researchers.[3,4] This lesion accounts for less than 3% of papillary urothelial neoplasm.[5] The majority of patients with a bladder tumor is diagnosed with superficial (i.e. pTa-pT1) disease at first presentation. High-grade carcinomas (G3), if still superficial, are associated with a poor prognosis and therefore these lesions require meticulous urological follow up and adjuvant treatment.[6] Conversely, G1 and a large percentage of G2 tumors (1973 WHO classification) behave rather indolent. For this reason, the 1998 WHO/ISUP classification system introduced new categories for papillary, non-invasive lesions of low cytologic grade to distinguish by means of strict morphological criteria between papillary urothelial neoplasms of low malignant potential (PUN-LMP) and low-grade papillary urothelial carcinoma (LG–PUC).[2]

The fibroblast growth factor receptor 3 (FGFR3) is part of a family of four highly conserved and structurally related tyrosine kinase receptors (FGFR1-4). The FGFRs are involved in cell signalling pathways regulating embryonic growth,

cell proliferation, angiogenesis, development and differentiation.[7] The human *FGFR3* gene, located on chromosome 4p16.3, consists of 19 exons and 18 introns.[8] Activating point mutations in the *FGFR3* gene are well documented in several craniosynostoses and skeletal dysplasias associated with dwarfism, including thanatophoric dysplasia (TD) and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN).[9,10] Next to the inhibitory role of *FGFR3* mutations in skeletal anomalies, an oncogenic role for *FGFR3* has been proposed in some malignancies. The same activating point mutations that accounted for the above mentioned skeletal anomalies were found in multiple myeloma [11] and the carcinomas of the bladder and the cervix.[12] The frequency of mutations in multiple myeloma and cervix cancer is probably lower than initially reported.[13-15] In addition, several malignancies and tumor cell lines were analysed for *FGFR3* mutations, but no mutations were found in other epithelial or other tumor types of various organs.[15,16] By contrast, in urothelial cell carcinoma (UCC), frequent mutations have been found in larger series.[17,18] Surprisingly, the presence of these mutations in UCC was related to favourable disease characteristics with up to 84% of cases with *FGFR3* mutations in pTaG1 tumors as compared to 7% in ≥pT2G3 tumors.[18]

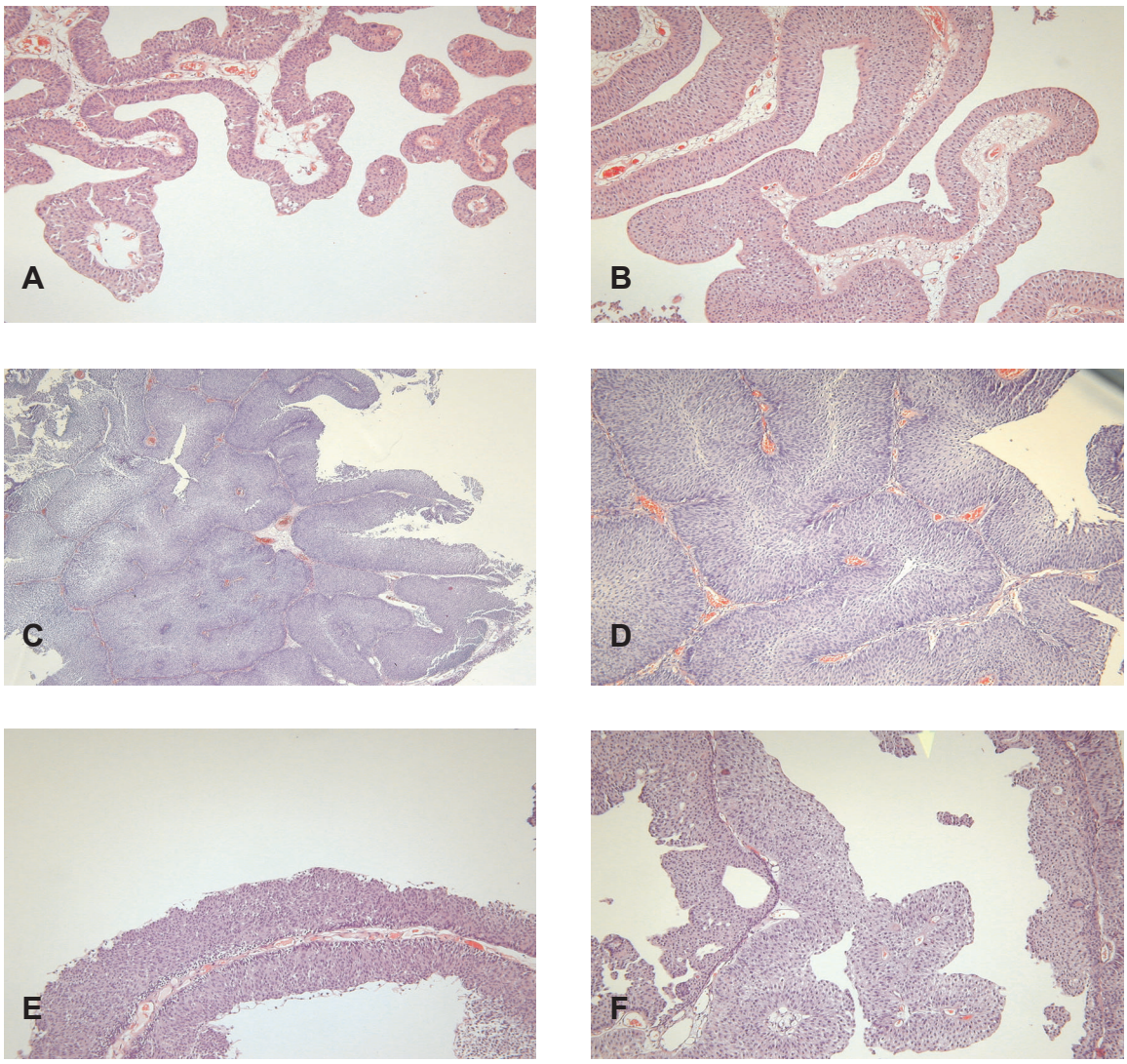
In the present study, we investigated the incidence of *FGFR3* mutations in papilloma, PUN-LMP and LG-PUC of the bladder. We also performed *FGFR3* mutation analysis in ovarian Brenner tumors of borderline malignancy because of their morphological resemblance to urothelial neoplasms.

MATERIAL AND METHODS

This study comprised 12 cases of urothelial papilloma and 79 pTaG1 tumors of 91 patients, graded according to the 1973 WHO classification for urothelial neoplasms[1] and staged according to the 1997 TNM classification guidelines.[19] Five papillomas were collected from the archives of the Department of Pathology of Erasmus University Rotterdam; four papillomas from the Institute of Pathological Anatomy and Histopathology, University of Ancona and three papillomas from the Department of Pathology of Sint Franciscus Gasthuis Rotterdam. The pTaG1 tumors were obtained from the two Departments of Pathology at Rotterdam. The histology slides of the urothelial papillomas and the pTaG1 tumors were reviewed (TvdK) according to the diagnostic criteria for urothelial papillary lesions of the 1998 WHO/ISUP classification system.[2] Histological examples of the different grades assessed are given in Figure 1. The bladder tumor cases were primary (first diagnosis) papillary bladder lesions. None had a coexistent or prior history of urothelial dysplasia, carcinoma in situ, invasive UCC, upper urinary tract UCC or, in case of multifocality, a tumor, which was >pTaG1. DNA extracted from venous blood for control purposes was available from 25 of 48 patients who were treated at the University hospital Rotterdam. Besides the bladder lesions, twenty-one ovarian Brenner tumors of borderline malignancy were obtained from the archives of the Department of

Pathology of Erasmus University Rotterdam. Standard H&E slides were made of the 112 paraffin-embedded tissue samples for pathological review. They also served as templates for the microdissection procedure to avoid contamination of the DNA sample with regions of normal mucosa, leukocytes or stroma.

Figure 1



Morphological examples of urothelial papilloma, papillary urothelial neoplasm of low malignant potential and low grade papillary urothelial carcinoma in the studied patients graded according to the 1998 WHO/ISUP classification system.[2] Examples A and B are urothelial papillomas characterised by a discrete papillary growth pattern with delicate fibrovascular cores lined by cytologically and architecturally normal urothelium. Examples C and D concern a papillary urothelial neoplasm of low malignant potential. This lesion shows an orderly proliferation of cohesive urothelial cells with normal polarity and without significant atypia. The papillae are lined by urothelium that is more than 7 cell layers in thickness. Examples E and F are low-grade papillary urothelial carcinoma. These are characterised by an overall orderly appearance but with easy recognisable variation of architectural and cytologic features, even at low power magnification. More detailed information on the 1998 WHO/ISUP classification system can be seen at: www.pathology.jhu.edu/bladder.

The samples used for *FGFR3* mutation analysis contained a minimum of 75% tumor cells, as assessed by histological examination. The DNA of the various tissues was extracted using DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) according to the protocol suggested by the manufacturer. The *FGFR3* mutation analysis was performed by polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis on tumor- and, if available, control DNA of the patients as described.[12,17,18]

In brief, four regions of interest encompassing all activating *FGFR3* mutations previously described in severe skeletal dysplasias and cancers were amplified by PCR. The Primer-sequences for PCR are shown in Table 1. 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. The laboratory analysis of the samples was carried out without the knowledge of clinical or histo-pathological status. Clinical information and follow up data were obtained by chart review. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for data documentation and analysis. The Chi-square test was used to analyse the *FGFR3* mutation status in relation to pathological assessment. The Chi-square test and ANOVA (for comparison of means) were used for comparison clinical and pathological variables with urothelial papilloma, PUN-LMP and LG-PUC. Statistical significance was assumed if $P<0.05$.

Table 1
The primer sequences for FGFR3 mutation analysis.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')
7	AGTGGCGGTGGTGGTGAGGGAG	TGTGCGTCACTGTACACCTTGCAG
10	CAACGCCCATGTCTTTGCAG	CGGGAAGCGGGAGATCTTG
15	GACCGAGGACAACGTGATG	GTGTGGGAAGGCGGTGTTG
19	TGTCGGCGCCTTTCGAGCAGTA	AGCAGCAGGGTGGGCTGCTA

RESULTS

The incidence of *FGFR3* mutations was studied in 12 papillomas, 62 PUNs-LMP and 17 LG-PUCs. We also analysed 21 Brenner tumors of borderline malignancy. The patient and tumor characteristics at diagnosis for the various types of papillary bladder tumors are reported in Table 2. These data show that papillomas are small, solitary papillary lesions. In addition, although significance was not reached because of the relative low number of papilloma patients, the papilloma patients were younger at diagnosis and the male-to-female ratio was 1.4:1 for papilloma and 2.8:1 for the pTaG1 tumors. The mean patient's age at diagnosis was 53.0 years (std. deviation, 9.5 years) for the Brenner tumor patients.

Table 2

*The patient and bladder tumor characteristics. The studied population consisted of 12 urothelial papillomas and 79 pTaG1 bladder carcinomas.[1,19] **Between parentheses: The bladder carcinomas were re-graded by one pathologist according to the 1998 WHO/ISUP classification.[2] ^ANOVA test. *Chi-square test.*

		Papilloma	pTaG1 (PUN-LMP, LG-PUC)**	P-value
Age (years)	mean	59.5	64.7 (65.5, 61.9)	
	std. deviation	15.4	12.5 (12.8, 11.2)	=0.194^
Sex	Male	7	58 (46, 12)	
	Female	5	21 (16, 5)	=0.281*
Multiplicity	Solitary	12	67 (54, 13)	
	Multiple	0	12 (8, 4)	=0.147*
Localisation in bladder	bladder neck	3	3 (2,1)	
	anterior wall	1	8 (6, 2)	
	dome	0	8 (8, 0)	
	posterior wall	2	9 (5, 4)	=0.174*
	trigone/ostia	2	13 (11, 2)	
	Left lateral wall	2	14 (12, 2)	
	right lateral wall	2	13 (11, 2)	
	multiple	0	11 (7, 4)	
Tumor size (diameter)	< 1cm	9	12 (12, 0)	
	1-3 cm	3	36 (31, 5)	<0.001*
	> 3 cm	0	31 (19, 12)	
Total		12	79 (62, 17)	

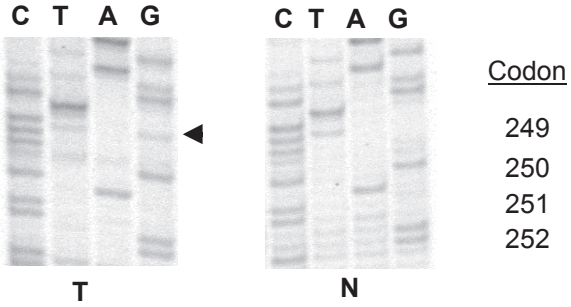
We detected five different shifts at SSCP analysis of the *FGFR3* gene. In total, 77 shifts were found. All the shifts were found among the 91 bladder neoplasms and none in the Brenner tumors. Sequence analysis revealed six different mutations in bladder neoplasms. These mutations concerned 5 different codons of the *FGFR3* gene (Table 3). The two different mutations in codon 652 displayed identical shifts at SSCP. The mutations in the codons 248, 249, 372 and 375 were reported before in TD and UCC.[9,12,17,18,20] The Lys652Met mutation was reported before in multiple myeloma and the germinal activating mutation is responsible for SADDAN.[9-11] The Lys652Thr was not previously reported in cancer or a syndrome associated with dwarfism. However, Bellus et al.[10] showed that this particular mutation is capable of FGFR3 activation (relative activity 3.1x that of wild type FGFR3). The Ser249Cys was the most frequent *FGFR3* mutation in bladder neoplasms accounting for 49 of the 77 mutations (64%). An example of this mutation in a bladder tumor is shown in Figure 2. Table 4 shows the distribution of *FGFR3* mutations among various bladder neoplasms. Interestingly, we found 9/12 (75%) *FGFR3* mutations in urothelial papillomas. A similar high percentage of *FGFR3* mutations was found in PUN-LMP and LG-PUC (P=0.589). There were no differences in the types of *FGFR3* mutations between the bladder neoplasms (not shown). No mutations were found in matched normal (blood) DNA samples from 25 bladder tumor patients confirming the somatic nature of *FGFR3* mutations in neoplasms.

Table 3

The FGFR3 mutations in bladder neoplasms. The codon and nucleotide numbering refers to the cDNA open reading frame of the FGFR3b isoform. This isoform is expressed in epithelia and contains two amino acids more than the FGFR3c isoform that is, in general, expressed in bone.[12]

Exon	Codon	nt Position	Mutation	Predicted effect	Number of tumors
7	248	742	CGC → TGC	Arg → Cys	7
7	249	746	TCC → TGC	Ser → Cys	49
10	372	1114	GGC → TGC	Gly → Cys	5
10	375	1124	TAT → TGT	Tyr → Cys	14
15	652	1955	AAG → ACG	Lys → Thr	1
15	652	1955	AAG → ATG	Lys → Met	1

Figure 2



An example of the most frequent FGFR3 gene mutation in bladder neoplasms. The G to C transition at nucleotide position 746 leading to the amino acid change Serine to Cysteine at codon 249 was found in 64% of mutated bladder neoplasms. The FGFR3 mutation in tumor is indicated by a solid arrowhead.
T = Sequence analysis of tumor DNA.
N = Sequence analysis of matched normal (blood) DNA.

Table 4

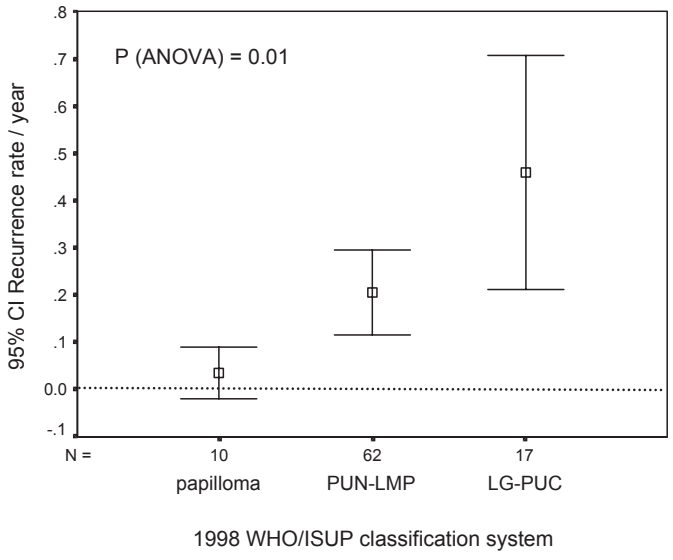
The FGFR3 mutation status in papillomas and pTaG1 tumors (1973 WHO classification) that were reviewed with the histological criteria of the 1998 WHO/ISUP classification system. The percentage of FGFR3 mutations found in each histological entity is between parenthesis.
**Chi-square test.*
PUN-LMP = Papillary Urothelial Neoplasm of Low Malignant Potential.
LG-PUC = Low Grade Papillary Urothelial Carcinoma.

	FGFR3 mutation analysis			
	Wild type	Mutant	% Mutant	P value*
Papilloma	3	9	75%	
PUN-LMP	9	53	85%	= 0.589
LG-PUC	2	15	88%	
Total	14	77	85%	

Follow up data were collected for the bladder tumor patients. Mean follow up was 4.84 years (range, 0.21-17.60 years) for the papilloma patients and 5.93 years (range, 1.11-16.26 years) for the patients initially diagnosed with pTaG1

(1973 WHO classification). Nine of the 12 papilloma patients remained recurrence free. One patient had recurrent papilloma as highest grade, one patient developed PUN-LMP and another one high-grade papillary urothelial carcinoma (HG-PUC) after a 6 years recurrence free period. No patients progressed to invasive (i.e. ≥pT2) bladder carcinoma or died of bladder cancer. Of the 62 patients with primary PUN-LMP, 33 had no recurrence, 19 had recurrent PUN-LMP, eight developed LG-PUC and 2 HG-PUC after 5½ and 12 years, respectively. One patient (1.6%) progressed to invasive disease and subsequently died of bladder cancer. Of the 17 patients with primary LG-PUC, 5 had no recurrence, 1 recurrent PUN-LMP, 9 recurrent LG-PUC and 2 developed HG-PUC after 2½ and 6 years, respectively. One patient (5.9%) progressed to invasive disease and subsequently died of bladder cancer. Interestingly, three of the 5 patients who developed HG-PUC had no *FGFR3* mutation (wild type) in their original tumor. Thus, 3/14 (21.4%) patients with wild type *FGFR3* tumors progressed to HG-PUC as compared to 2/77 (2.6%) for mutant *FGFR3* tumors (P=0.025). To further explore the long-term natural behaviour of urothelial papilloma, PUN-LMP and LG-PUC, we calculated the recurrence rate per year (number of recurrences divided by years of follow up) for each histological category. Minimum follow up was set at one year, so two papilloma patients were excluded from this analysis. The mean (95% confidence interval for mean) recurrence rate per year was 0.03 (-0.02 – 0.09) for the papilloma patients, 0.21 (0.11 – 0.30) for the PUN-LMP patients and 0.46 (0.21 – 0.71) for the LG-PUC patients (Figure 3).

Figure 3



The recurrence rate per year (number of recurrences / years of follow up) was calculated for each histological category. The bars represent the 95% confidence intervals for the mean. The figure shows a different natural clinical behaviour using the 1998 WHO/ISUP classification system for the 3 histological tumor types (P value for comparison of means (ANOVA) = 0.01). PUN-LMP = Papillary Urothelial Neoplasm of Low Malignant Potential. LG-PUC = Low Grade Papillary Urothelial Carcinoma.

Taken together, these follow up data point to a spectrum of disease outcome for cases classified according to the 1998 WHO classification system from a relative

indolent natural behaviour of papilloma towards a more aggressive behaviour of LG-PUC.

DISCUSSION

Urothelial papillomas of the bladder are rare and generally considered benign lesions since these patients seldomly experience recurrence or development of UCC [4,5]. Furthermore, papillomas usually are small (< 2 cm), solitary lesions which are generally diagnosed at a somewhat lower age and occur relatively more often than UCC in women compared to men.[2-4,21] Apart from one papilloma patient who developed high-grade disease, our series of 12 papillomas closely resembled the above mentioned papilloma characteristics. These favourable characteristics justify that papillomas are a distinct entity in the classification systems used by pathologists.[1,2] However, the high frequency (75%) of oncogenic *FGFR3* mutations in papillomas reported in this paper suggests that papillomas should be grouped together with the well differentiated papillary urothelial neoplasms which now range from PUN-LMP to LG-PUC.

The oncogenic role of *FGFR3* mutations in urothelium was recently studied.[12,17,18,20] The highest frequency of *FGFR3* mutations was detected in pTaG1 carcinomas, comparable with the incidence of mutations reported in the present study.[17,18] In addition, we found that the presence of a *FGFR3* mutation was associated with a low recurrence rate of superficial bladder cancer.[17] This prompted us to analyse the *FGFR3* mutation status in papillomas. Three studies analysed the expression patterns of immuno-histochemical markers (CK20, CD44, TP53, Ki-67/MIB-1) in small series of in total 18 papillomas.[22-24] Essentially, it was not possible with these markers to discriminate normal from papilloma urothelium. In addition, Chow et al.[25] investigated 4 papillomas and 15 papillary hyperplasias (PH) for loss of heterozygosity (LOH) at 17 microsatellite markers on 9 chromosomal arms. LOH was found in 8/15 PHs but no genetic changes were found in the papillomas examined. Thus, to our knowledge, the present report is the first to describe a genetic defect (i.e. *FGFR3* mutation) in urothelial papilloma. Most LOHs in PH were detected on chromosome 9, confirming that LOH on chromosome 9 is an early event in human bladder tumorigenesis.[25-27] Chow et al.[25] concluded that PH was the clonal precursor to papillary UCC. As we now have identified a high percentage of *FGFR3* mutations in papillomas and low-grade UCC, this indicates that papilloma may also be regarded as a precursor of (low-grade) UCC. Moreover, papilloma and PH might represent two separate precursor pathways with distinct molecular features (i.e. *FGFR3* mutation in papilloma and LOH in PH). However, this should be interpreted with caution as Chow et al.[25] only investigated 4 papillomas and we did not analyse our papillomas for LOH. Next to genetics, clinical and pathological parameters of papilloma such as tumor size, solitary lesion, lower mean age at diagnosis also advocate its potential as precursor lesion for UCC. Furthermore, the rare association with development of UCC and the notion that

papillomas are usually successfully treated with a single surgical intervention[4] may indicate that papilloma is still a local neoplasm which is not capable of spread throughout the bladder as UCC.

Some studies have recently investigated the 1998 WHO/ISUP classification system for its ability to predict the natural course of bladder neoplasms and its relationship with the expression patterns of certain immuno-histochemical markers.[21,22,28,29] In these and the present study, the patients had a risk of recurrence ranging from 25% to 47% for PUN-LMP and from 48% to 71% for LG-PUC. The risk of progression to invasive (i.e. ≥pT2) UCC ranged 0% to 1.8% for PUN-LMP and from 4.0% to 6.8% for LG-PUC. These reports indicate that 1998 WHO/ISUP classification system more or less parallels the one of 1973 for the group of tumors analysed in this study (i.e. PUN-LMP ≈ Grade 1 carcinoma and LG-PUC ≈ Grade 2 carcinoma). Anyhow, the adherence to the strict morphological criteria defined in 1998 may lead to a better prediction of the natural course of bladder tumors because patients with an original diagnosis of pTaG1 who were "upgraded" by the 1998 WHO/ISUP classification system to LG-PUC had a worse natural course than the patients with PUN-LMP in our study (Figure 3). However, we have to take into account that the original diagnosis (pTaG1) was provided by many different pathologists in a routine setting and, of course, grading is subject to inter- and intra-observer variability.[30] A criticism to the 1998 WHO/ISUP classification and previous classification systems was that the morphological approach was not complemented with the use of molecular markers.[31] If molecular markers prove to be of independent significance, the elimination of subjective elements in grading by adding these markers holds considerable promise. A normal CK-20 staining pattern was related to tumors, which were less likely to recur.[22,29,32] The *FGFR3* mutation also identified patients with tumors, which were less likely to recur.[17] However, identification of *FGFR3* mutations has two additional advantages. As it is not determined by immuno-histochemistry, interobsever variability is not possible and secondly, the *FGFR3* mutation is not present in normal-looking random urothelial biopsies from patients known to have a *FGFR3* mutation in their papillary UCC.[18] Therefore, the *FGFR3* mutation and its apparent positive identification of low risk patients represent a major target for future UCC research.

Having found frequent (75%) *FGFR3* mutations in urothelial papillomas, we chose to further analyse the *FGFR3* mutation status in another epithelial tumor. Our choice for Brenner tumors of the ovary was supported by the resemblance of this tumor to low grade UCC from a cytological point of view. Moreover, expression of uroplakins, CK13 and CK20 was detected in Brenner tumors as well as in normal and neoplastic urothelium.[33,34] Nevertheless, we found no *FGFR3* mutations in this form of borderline malignancy. Therefore, *FGFR3* mutations seem to be very specific for urothelial neoplasms as reported.[15,16]

The *FGFR3* mutation is found at a high frequency in “favourable” urothelial neoplasms and carcinomas. In addition, this mutation is also present in the majority of urothelial papillomas. The strict morphological criteria defined in the 1998 WHO/ISUP classification system for papillary neoplasms of the bladder encomprise a spectrum of disease outcome from favourable (papilloma) towards a more aggressive behaviour of LG-PUC. From a molecular perspective, urothelial papillomas should subsume the spectrum of well-differentiated urothelial neoplasms.

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11

FGFR3 and *P53* Characterize Alternative Genetic Pathways in the Pathogenesis of Urothelial Cell Carcinoma

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ABSTRACT

Fibroblast growth factor receptor 3 (*FGFR3*) and *P53* mutations are frequently observed in urothelial cell carcinoma (UCC). We here describe the distribution of *FGFR3* and P53 over-expression in 260 primary bladder cancers. *FGFR3* mutations were observed in 59% and P53 over-expression in 25%. Interestingly, *FGFR3* and P53 alterations were mutually exclusive as they coincided in only 5.7% of tumors. Consequently, we propose that they characterize two alternative genetic pathways in UCC pathogenesis. The genetic alterations were reflected in the pathology and the clinical outcome, i.e. *FGFR3* mutations were found in low stage/grade tumors and were associated with a favorable prognosis, whereas P53 alterations were tied to adverse disease parameters.

INTRODUCTION

Urothelial cell carcinoma (UCC), of which bladder cancer is the major representative, is the fifth most common malignancy in the western society.[1] Because of its frequent recurrence and the relatively long life span of patients, UCC is the most expensive cancer in health care.[2] Approximately 70% of the patients with UCC initially present with superficial tumors (stages: pTa, pT1 or pTis). UCCs fall into two major groups with a substantially different natural behavior, i.e. superficial and invasive UCC. More than 80% of superficial UCCs remain confined to the (sub)mucosa throughout their clinical course, whereas most invasive UCCs exhibit their invasive property at first presentation and these tumors are associated with a high propensity to metastasize.[3] In the last decade, many efforts were undertaken to find a molecular basis for this divergent disease pathogenesis of UCC. Mutations in the *P53* gene were frequently found in invasive UCC as well as high-grade superficial UCC including carcinoma in situ (CIS), the putative precursor of invasive UCC, whereas these mutations were rare in well-differentiated superficial UCC.[4,5] On the other hand, loss of heterozygosity (LOH) on the chromosomal arms 9p and 9q was the most frequent genetic alteration in the low-grade papillary lesions.[4,5] However, subsequent studies showed that LOH on 9p/q was also a very common finding in high grade superficial and invasive UCC.[6-9] Consequently, LOH on 9p/q did not provide a sufficient molecular explanation for the clinically divergent disease pathogenesis of UCC. Recently, *fibroblast growth factor receptor 3 (FGFR3)* mutations, identical to the mutations responsible for several skeletal anomalies associated with dwarfism in most cases, were reported at a high frequency in UCC and at a low frequency in multiple myeloma and cervical cancer.[10-12] Surprisingly, the oncogenic *FGFR3* mutations were particularly related to favorable UCCs in three pilot studies.[13-15] The *FGFR3* mutations occur predominantly in UCC [11,12], while *P53* mutations are found in over 50% of human cancers. In the present study, we investigated the distribution of *FGFR3* and *P53* alterations in 260 primary (first diagnosis) UCCs. We here report that *FGFR3* and

P53 characterize almost 80% of UCCs and that these mutations seem to exclude each other. These distinct molecular features were also reflected in the different pathological parameters and the clinical follow-up of the patients. We therefore propose that *FGFR3* and *P53* characterize different pathogenesis pathways for UCC.

MATERIAL AND METHODS

Patients & Tumor Samples. We analyzed the tumors of 260 patients (196 males) with papillary, first diagnosis, UCC. The median patient's age was 67.2 years. No patient had a hereditary skeletal anomaly. A paraffin-embedded, formalin fixed tissue block was obtained from the archives of two pathology departments (Erasmus MC and SFG) and classified according to the TNM and WHO guidelines. A single pathologist (TvdK) reviewed the slides using the 1998 WHO/ISUP classification system for grading. In case of multifocality (N=67), the lesion with the highest grade/stage was taken. The largest tumor was taken if grade/stage were the same for multiple UCCs.

***P53* analysis:** Four μm thick sections were freshly cut from each tumor-tissue block. These were mounted on amino alkylsilane coated glass slides. Incubation with primary antibody P53 (clone DO-7, DAKO, Denmark, dilution 1:200) was 30 min in PBS/BSA 5%. Positive and negative controls were included. The conventional avidin-biotin complex method was applied for all immuno-stainings. Two persons (BvR, AV) independently assessed the slides without knowledge of clinical data. In case of heterogeneity, the parts within the tumor that showed highest positive to total ratio were particularly assessed. This was performed if these regions comprised at least 10% of the tumor load in the examined tissue section. P53 over-expression was scored if >10% stained positive. In case of discrepancy between the observers, the slides were reassessed in a combined session without the information of the previous scores.

***FGFR3* analysis:** Standard H&E slides served as templates for manual micro-dissection. The dissected samples contained a minimum of 70% tumor cells, as assessed by histological examination. The DNA was extracted using the DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany). The *FGFR3* mutation analysis was performed by polymerase chain reaction (PCR) - single strand conformation polymorphism (SSCP) analysis.[14] Samples with a shift were sequenced with T7 Sequenase v2.0 (Amersham life Science, Inc., Cleveland, OH). In addition, DNA extracted from venous blood was available from the 139 patients who attended the Urology department of the Erasmus MC. These laboratory analyses were also performed without knowledge of clinical data.

Clinical Follow-up & Statistical analysis. The follow-up data were collected by chart review. Disease specific survival was determined. The patients were censored at their last clinical visit or the time of their death. The statistical package for social sciences 9.0 (SPSS Inc., Chicago, IL) computer software was used for the data documentation and analysis. The two-sided Fisher's exact test

was used to analyze the relations between the molecular variables and their correlation with pathological stage and grade. The clinical outcome was analyzed by applying the Kaplan-Meier method. Statistical Significance was assumed if $P<0.05$.

RESULTS

Mutations in the *P53* and *FGFR3* genes were studied in 260 primary UCCs. Activating *FGFR3* mutations were found in 153 (59%) of these tumors. We found 117, 32 and 4 mutations in the exons 7 (R248C & S249C), 10 (G372C, Y375C & A393E) and 15 (K652T, K652E & K652M), respectively. No mutations were detected in exon 19. No activating *FGFR3* mutations were seen in the matched blood samples. P53 over-expression, presumably reflecting missense mutations in the *P53* gene, was noted in 66 (25%) cases. Table 1 shows a highly significant inverse relation between the presence of a *FGFR3* mutation and P53 over-expression ($P < 0.0001$). Only 5.7% of the 260 primary UCCs were positive for both molecular features whereas 72.7% UCCs were either positive for *FGFR3* or positive for P53.

Table 1

Inverse relationship between FGFR3 mutations and P53 over-expression in Urothelial Cell Carcinoma (P-value < 0.0001). A P53 mutation was assumed if >10% of the cells stained positive.

	FGFR3 wt	*FGFR3* mt
P53 wt	56	138
P53 mt	51	15
Total	107	153

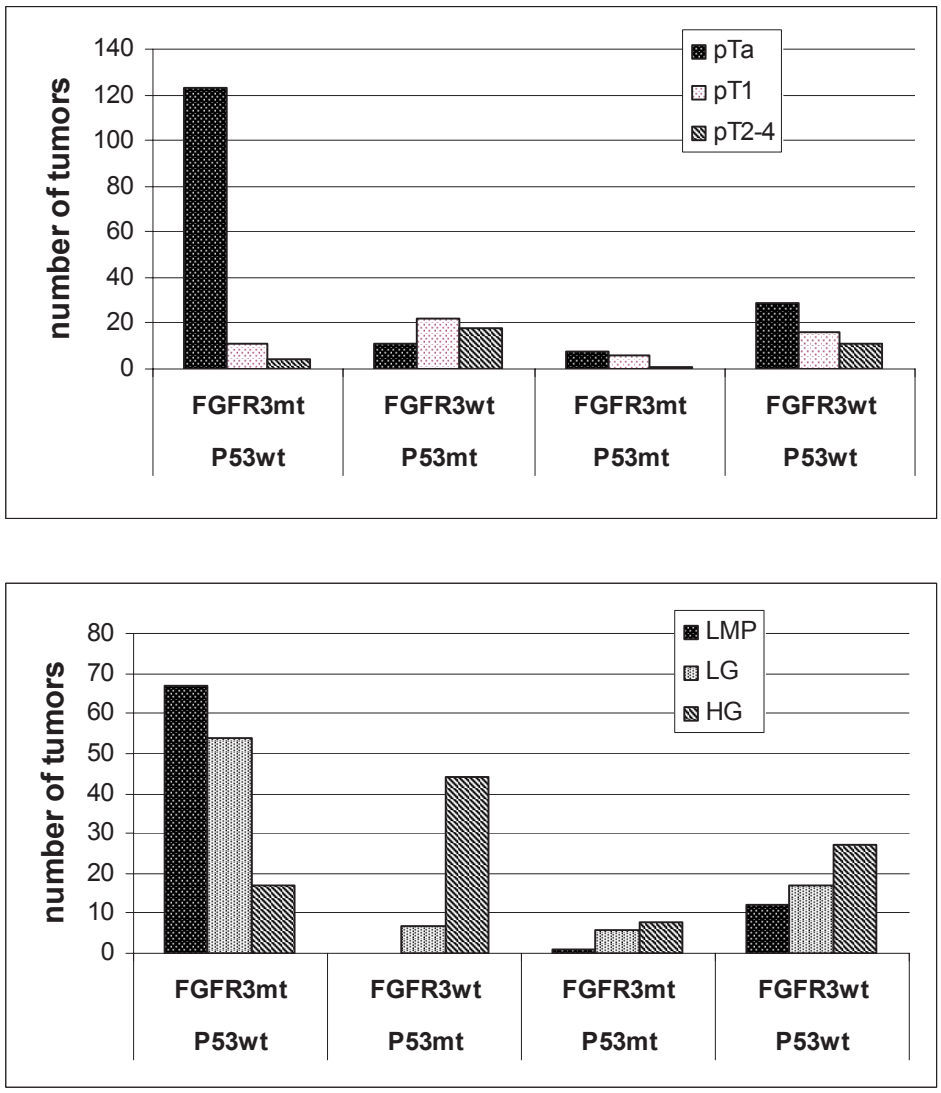
Abbreviations: wt, wild type; mt, mutated type; FGFR3, fibroblast growth factor receptor 3.

We subsequently determined the correlation of *FGFR3* and *P53* with pathological Stage and Grade for the 260 UCCs. *FGFR3* mutations were found in 77% of pTa, 31% pT1 and 15% ≥pT2 tumors. Conversely, P53 over-expression was found in 11% pTa, 51% pT1 and 56% ≥pT2 tumors, respectively (P-values < 0.0001). The numbers for pathological Grade showed the same trend. *FGFR3* mutations were found in 85% of urothelial neoplasia of low malignant potential (LMP), in 71% of low grade papillary urothelial carcinoma (LG) and in 26% of high grade papillary urothelial carcinoma (HG) cases. On the other hand, P53 over-expression was found in only 1% of LMP, in 15% of LG and in 54% of HG cases, respectively (P-values < 0.0001). The distributions of the various pathological Stages and Grades for the *FGFR3*/P53 subgroups are given in Figure 1. In general, the *FGFR3* mutation related to favorable, i.e. pTa and LMP/LG disease, whereas P53 over-expression indicated unfavorable, i.e. invasive and HG disease.

The UCCs, which were wild type for both genes, the so-called double negatives, were a substantial subgroup encompassing 21% of the 260 UCCs

(Table 1). When compared to Stage and Grade, these UCCs apparently comprised tumors of all grades and stages, with a higher percentage pTa tumors than the P53-positive subgroup, i.e. 51% vs. 22%, but also a higher percentage high Grade tumors than the *FGFR3*-mutation subgroup, i.e. 48% vs. 12% (Figure1).

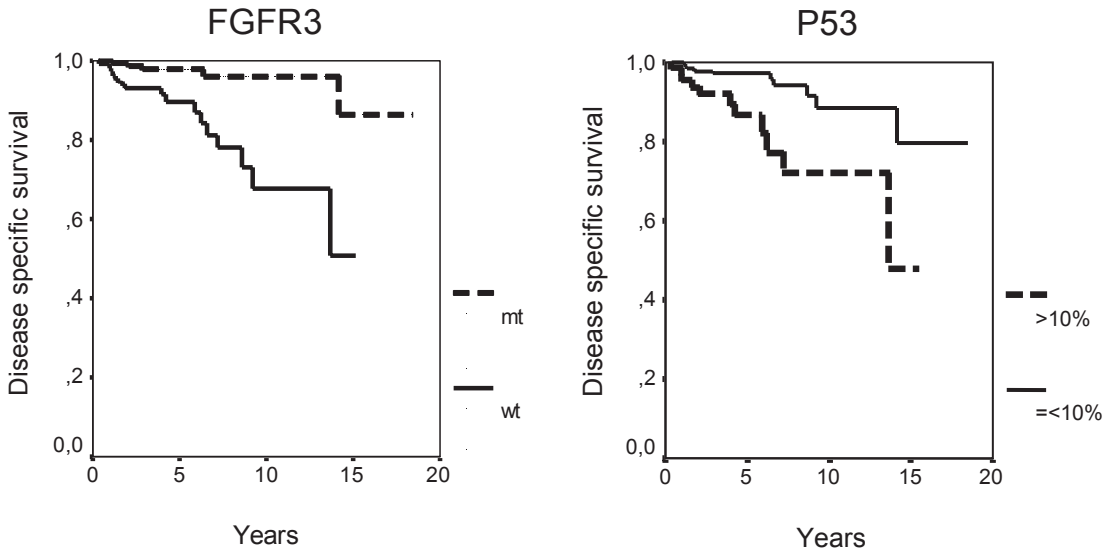
Figure 1



The Relation for FGFR3/P53 subgroups with pathological Stage (upper panel) and Grade (lower panel). The Stage distribution was 171 pTa, 55 pT1 and 27 pT2 and 5 pT3 and 2 pT4 lesions. Note the correlation between mutations in the FGFR3 gene with pTa and low grade (LMP & LG) tumors. Conversely, P53 over-expression, presumably caused by missense mutations, was associated with invasive (≥ pT1) and high-grade (HG) disease. The tumors that were wild type for both genes represent a group, in which all stages and grades are found.

Besides histopathological parameters, the clinical outcome of the 260 patients was also determined. The mean follow-up was 5.6 years (standard deviation: 3.7 years). Twenty-one patients died of UCC. The Kaplan-Meier analyses in Figure 2 clearly show that patients with a *FGFR3* mutation in their UCC have a favorable prognosis, whereas patients with P53 over-expression have a worse prognosis than patients with a normal P53 expression pattern.

Figure 2



Kaplan-Meier analyses for disease specific survival. The survival plots (log-rank) for FGFR3 (P=0.0001) and P53 (P=0.0011) are shown. The dashed lines display the follow-up of patients with a FGFR3 mutation and P53 over-expression, respectively.

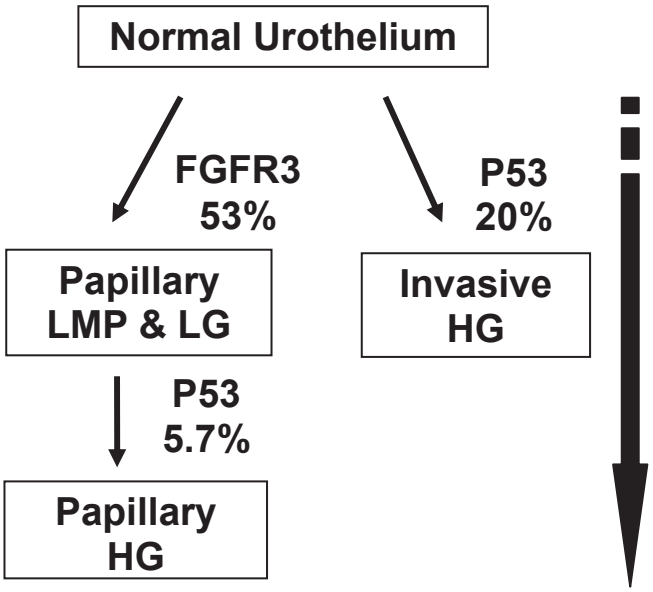
DISCUSSION

UCCs are genetically characterized by frequent losses of heterozygosity (LOH), mutations and deletions in the *P53, RB* and *p16* tumor suppressor genes and activating point mutations in the gene for the FGFR3. LOH of chromosomal arms 9p and 9q is observed in over 50% of UCC. Homozygous deletions of the p16 cyclin dependent kinase inhibitor are found in 20-30% of high grade and stage tumors.[16] Bladder tumors with pRB alterations are significantly more prone to metastasize (ref. 17 and references therein). Mutations in the *P53* gene are observed in poorly differentiated carcinomas and mutations in this gene apparently characterize tumors with a worse prognosis. Based on these observations Spruck et al.[5], proposed a two-pathway model for UCC pathogenesis, in which *P53* mutations delineated one arm of the pathway. LOH on chromosome 9 was supposed to characterize the other arm. However, later work revealed that chromosome 9 loss is found in all grades and stages and therefore can no longer serve as a marker for the non-invasive pathway.[6-9] A recent update of the UCC pathogenesis model, including more markers, was

published by Cote and Datar.[17] However, although many genes may play a role in UCC pathogenesis, most of them are only mutated in a limited percentage of tumors and, at present, their mutual dependencies, when present, are not entirely clear.

We have recently shown that *FGFR3* mutations are generally associated with favorable disease characteristics such as low stage and grade, low recurrence rate and a better prognosis.[13,18] In the present study, we intended to place this new genetic marker in the genetic pathogenesis model for UCC. To this end, we determined the *FGFR3* and *P53* status in 260 primary tumors. To our surprise, we observed that alterations in *FGFR3* and *P53* were almost always mutually exclusive. Together these two markers described 79% of the primary UCC. However, they concurred in only 5.7%. This suggested that *FGFR3* and *P53* characterize two distinct genetic pathways in UCC pathogenesis. While this paper was being reviewed, Bakkar et al. reported similar findings based on a study of 81 patients.[19] The *FGFR3* mutation resulted in pTa and LMP/LG tumors, whereas the P53 pathway led to invasive and HG carcinomas. In addition, the clinical outcome of the two investigated molecular markers confirmed this suggestion of two genetic pathways. A model for UCC pathogenesis based on our observations is given in Figure 3.

Figure 3



Model for UCC pathogenesis. FGFR3 and P53 indicate two different pathways for UCC pathogenesis and together they classify 79% of primary UCCs. These mutations clearly result in distinct pathological parameters. The small group of tumors with mutations in both genes is presumably derived from the FGFR3 group (see also text). The 21% tumors that lack alterations in P53 or FGFR3 are not included in the figure. The arrow on the right signifies increasing LOH and mutations in other genes.

This model resembles Spruck's original model, but clearly designates two distinct pathways with genetic markers for a non-invasive and papillary, FGFR3-associated pathway and an invasive P53-associated pathway. Together, these two markers provided a genetic framework for the majority of UCCs. The addition of the *FGFR3* marker significantly expands Spruck's original model (most recently reviewed in Cote and Datar[17]). We feel that, at the moment, it is not completely clear where to put LOH, especially of chromosome 9q, in this pathway. In *P53*-positive tumors, this event may have occurred before the *P53* mutation. However, in a considerable percentage of *FGFR3*-mutant tumors, LOH may occur after the *FGFR3* mutation, since *FGFR3* mutations are more frequent than LOH of 9q. On the other hand, we previously showed in evolutionary tree models of multiple UCC recurrences that tumors with LOH of 9q can precede recurrences with a mutation in the *FGFR3* gene.[7] Thus, the *FGFR3* mutation is not necessarily the first genetic event in superficial tumor formation. In the model displayed here, we also suggest that UCC with alterations in both *FGFR3* and *P53* derive from *FGFR3*-mutant tumors. We argued that it would be more difficult for a *P53* positive, invasive, HG carcinoma to become a lower grade superficial tumor than that a pTa, LMP/LG tumor transforms into a more invasive, higher grade descendant. This small group of UCCs apparently displays an intermediate phenotype when compared to the tumors with single alterations (ref. 18 and present study). Thus, it appears that the effect of the *FGFR3* mutation mitigates the effect of the *P53* mutation. The remainder of UCCs, 21% in our series, lacking *P53* or *FGFR3* alterations forms an interesting subset. The fact these tumors comprise all possible grades and stages suggests that they may also harbor different genotypes. Some of these tumors may in the future perhaps be placed in an *FGFR3*-like group or a *P53*-like group. However, it is also possible that yet a third genetic pathway is responsible for a portion of these tumors. Previous work reviewed by Cote and Datar [17] suggests that RB and p16 and/or pARF alterations are mostly associated with the P53-mutant UCCs and, thereby, are expected to be part of this pathway. However, it is not yet clear whether these mutations occur before or after the *P53* event. The same holds true for other less frequently occurring alterations. So far, no mutations, besides LOH for 9p/9q and other chromosomes have been described to concur with *FGFR3* mutations. Therefore, additional investigations are required in order to place all markers in the pathway model. Consequently, the arrow on the right in Figure 3 signifies an increasing frequency of LOH and other genetic aberrations from the top to the bottom.

The colorectal cancer model proposed by Fearon and Vogelstein is the prototype for the molecular evolution of cancer.[20] The model is presented as a linear model, in which inactivation of *APC*, mutation of *KRAS*, inactivation of a gene on 18q and inactivation of *P53* occur as subsequent steps in the development from low-grade adenomas to carcinoma. However, Smith et al. [21] recently showed that co-occurrence of mutations in both *KRAS* and *P53* in

colorectal cancers is extremely rare and they suggest that these mutations in fact lie on alternate pathways of colorectal tumor development. Thus, the route to colorectal cancer seems to bifurcate after the initial *APC* step into at least two alternative genetic pathways. This situation is similar to the one we describe for UCC in Figure 3. In colorectal cancer, *K-RAS* mutations are over-represented in Dukes C tumors and, thereby, it may be a marker for tumor progression. The added value of the model for UCC pathogenesis is that *FGFR3* and *P53* represent makers for favorable and unfavorable disease, respectively. Together, these markers characterize almost 80% of all primary tumors. The search for mutations in other genes of both pathways may increase the percentage of molecularly characterized tumors further and may facilitate a more complete molecular description of UCC as a possible alternative for classical pathology.[18] Moreover, the favorable *FGFR3* marker is a potential candidate to identify patients for whom a less frequent follow-up is required and this may help reduce the costs of management for patients with UCC.

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12

Novel *Fibroblast Growth Factor Receptor 3* (*FGFR3*) Mutations in Bladder Cancer Previously Identified in Non-Lethal Skeletal Disorders

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ABSTRACT

Activating mutations in the fibroblast growth factor receptor 3 (*FGFR3*) gene are responsible for several autosomal dominant craniosynostosis syndromes and chondrodysplasias i.e. hypochondroplasia, achondroplasia, SADDAN and thanatophoric dysplasia - a neonatal lethal dwarfism syndrome. Recently, activating *FGFR3* mutations have also been found to be present in cancer, i.e. at high frequency in carcinoma of the bladder and rarely in multiple myeloma and carcinoma of the cervix. Almost all reported mutations in carcinomas corresponded to the mutations identified in thanatophoric dysplasia. We here screened a series of 297 bladder tumors and found three *FGFR3* somatic mutations (G380/382R; K650/652M and K650/652T) that were not previously identified in carcinomas or thanatophoric dysplasia. Another novel finding was the occurrence of two simultaneous *FGFR3* mutations in 4 tumors. Two of the three new mutations in bladder cancer, the G380/382R and the K650/652M mutations, were previously reported in achondroplasia and SADDAN, respectively. These syndromes entail a longer life span than thanatophoric dysplasia. The K650/652T mutation has not previously been detected in patients with skeletal disorders, but affects a codon that has been shown to be affected in some cases of thanatophoric dysplasia, SADDAN and hypochondroplasia. From a clinical perspective, the patients with FGFR3-related, non-lethal skeletal disorders might be at a higher risk for development of bladder tumors than the general population.

INTRODUCTION

The fibroblast growth factor receptor 3 (FGFR3) belongs to a family of structurally related tyrosine kinase receptors encoded by four different genes (FGFR1-4). These receptors consist of three glycosylated extracellular immunoglobulin-like domains (Ig-like), a transmembrane domain and a split intracellular tyrosine-kinase domain. Ligand binding induces FGFR dimerization, resulting in autophosphorylation of the kinase domain and interaction with and phosphorylation of effector signaling proteins.[1,2] Alternative mRNA splicing mechanisms generate many different receptor isoforms, which differ in ligand specificity. The isoforms FGFR3b and FGFR3c result from a mutually exclusive splicing event, in which the second half of the third Ig-like domain is encoded by either the 151 nucleotides of exon 8 or the 145 nucleotides of exon 9.[3] These two isoforms have different tissue distributions: for example, FGFR3b is the main form in epithelial cells whereas FGFR3c is the predominant form in chondrocytes.[3-5]

Point mutations in specific domains of *FGFR3* are associated with autosomal dominant dwarfism and craniosynostosis syndromes such as hypochondroplasia, achondroplasia (the most common form of skeletal dysplasia), severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), thanatophoric dysplasia (a lethal form of dwarfism),

Crouzon syndrome with acanthosis nigricans and Muenke coronal craniosynostosis.[3,6,7] Several reports have demonstrated that these mutations lead to constitutive activation of the receptor.[8-11]

In contrast with the inhibitory role on bone growth, an oncogenic role for *FGFR3* in human cancer has emerged. Indeed, somatic activating mutations in *FGFR3* have been reported in multiple myeloma and, more recently, in two epithelial malignancies, i.e. bladder- and cervix carcinomas.[5,12,13] *FGFR3* mutations are rare in multiple myeloma and cervix carcinomas,[14,15] whereas their high incidence in bladder carcinomas (74% of non-invasive papillary tumors) suggests that the constitutive activation of FGFR3 is an important event for bladder tumorigenesis.[16,17] Nearly all mutations identified in bladder tumors are identical to the activating mutations responsible for thanatophoric dysplasia, a lethal form of dwarfism.[5,16-18] Only two of the 117 *FGFR3* mutations identified by these groups, the A393E and K652Q mutations, do not correspond to thanatophoric dysplasia mutations.[17,18] The A393E mutation is identical to a mutation associated with a craniosynostosis syndrome (Crouzon syndrome with acanthosis nigricans) and the K652Q mutation is identical to a mutation associated with hypochondroplasia.[11,19]

In this report, we describe three new somatic *FGFR3* mutations in bladder tumors (G380/382R, K650/652M and K650/652T) (FGFR3c isoform numbering/FGFR3b isoform numbering). The G380/382R and the K650/652M mutations have previously been reported in achondroplasia and SADDAN, respectively. The K650/652T mutation has not been reported before in a skeletal disorder. In addition, among the 177 mutated tumors, we observed the occurrence of two concurrent *FGFR3* mutations in 4 cases.

MATERIALS AND METHODS

FGFR3 mutation analysis was performed in a series of 297 bladder tumors as described.[16] T7 Sequenase v2.0 (Amersham life Science, Inc., Cleveland, OH) was used for sequencing to analyze the four regions of *FGFR3*, located in exons 7, 10, 15 and 19, known to harbor the point mutations previously described in multiple myeloma, bladder- and cervix carcinomas, thanatophoric dysplasia and SADDAN. None of the 297 patients had a hereditary skeletal disorder documented. The mean patient's age at diagnosis was 65.9 years (range, 23–90). Seventy (24%) of the 297 patients were female. The three new mutations of *FGFR3* and all the "double" mutations were confirmed on a second PCR product.

RESULTS

In this series of 297 bladder carcinomas, we detected 181 *FGFR3* mutations. Consistent with previous studies,[5,16-18] most of the mutations (173/181, 96%), were identical to the germinal activating mutations responsible for thanatophoric dysplasia, with the S249C mutation, found in 125 (69%) of the 181

mutations as the most frequent point mutation. The A391/393E mutation, identical to the germinal mutation that causes Crouzon syndrome with acanthosis nigricans and already identified in bladder cancer,[17] was found in four tumors.

We detected three mutations in this series of 297 bladder carcinomas, which were not previously identified in carcinomas or in thanatophoric dysplasia patients (Figure 1A and 1B).

Figure 1A

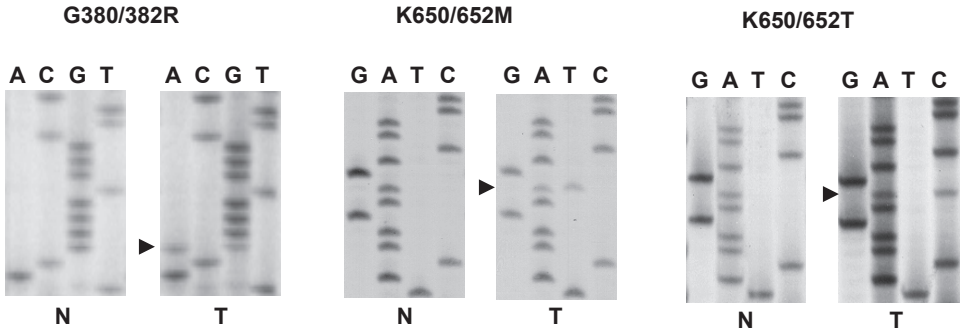
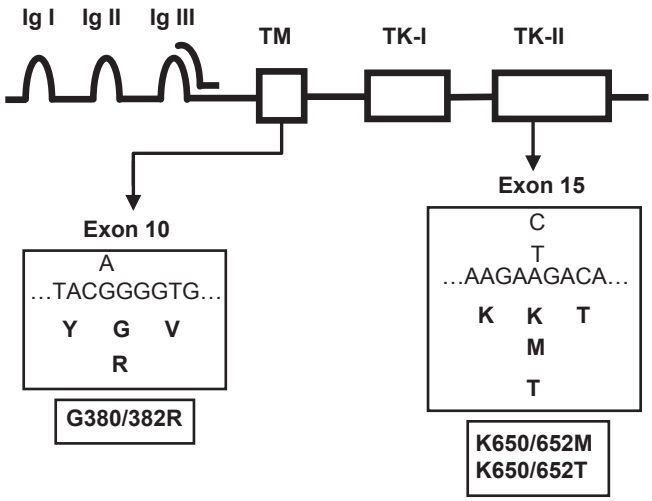


Figure 1B



Identification of novel FGFR3 mutations in bladder cancer.
Figure 1A. Novel somatic FGFR3 mutations were identified by PCR-SSCP followed by direct DNA sequencing. The tumor shown on the left side has a G to A transition in exon 10, which changes the sequence of codon 380/382 from GGG (Gly) to AGG (Arg). The tumor in the middle has an A to T transversion in exon 15, which changes the sequence of codon 650/652 from AAG (Lys) to ATG (Met). The tumor on the right side has an A to C transversion in exon 15, which alters the sequence of codon 650/652 from AAG (Lys) to ACG (Thr). Arrowheads indicate the positions of the mutations. The FGFR3b isoform, which is expressed in epithelial cells, contains two amino acids more than the FGFR3c isoform expressed in bone. Therefore, the G380R, K650M and K650T mutations in FGFR3c are equivalent to the G382R, K652M and K652T mutations in FGFR3b. Note in addition: while the present study was under evaluation, the K650/652M mutation in bladder cancer was reported by Kimura et al.[26] N, sequence of normal DNA; T, sequence of tumor DNA.
Figure 1B. Schematic diagram of the FGFR3 protein. The areas within the two exons, in which the new missense mutations occurred, are shown in greater detail. The nucleotide substitutions are shown above, the amino acid substitutions are shown at the bottom of the Figure.

These mutations affected the codons 380/382 and 650/652 (FGFR3c isoform numbering/FGFR3b isoform numbering). The G380/382R mutation affected the transmembrane domain and resulted in the replacement of a glycine by an arginine residue. This mutation is identical to a germinal activating mutation associated with the most common form of genetic dwarfism, i.e. achondroplasia.[20-22] The other two mutations (K650/652M and K650/652T), affecting the tyrosine kinase domain, resulted in the replacement of a lysine by a methionine or a threonine. The K650/652M mutation is identical to the germinal activating mutation that causes SADDAN.[23] This novel mutation was found twice in this series of tumors. The matched constitutional DNA contained the wild-type sequence in every case, demonstrating the somatic nature of these mutations in (bladder) cancer. The tumor with the G380/382R mutation also displayed a S249C mutation in *FGFR3*. This patient suffered a recurrence five years later and the recurrent tumor was found to contain the same two *FGFR3* mutations.

In addition to the above-mentioned case, three other bladder tumors also contained two distinct *FGFR3* mutations. In each case, the S249C mutation was present. S249C was accompanied by the R248C, G370/372C and A391/393E mutations. The clinico-pathological data of the bladder-carcinoma patients carrying the new and the "double" mutations are depicted together with the type of *FGFR3* mutations in Table 1. We found no significant differences in the pathological status or the clinical data for the various *FGFR3* mutations in bladder cancer (not shown).

Table 1

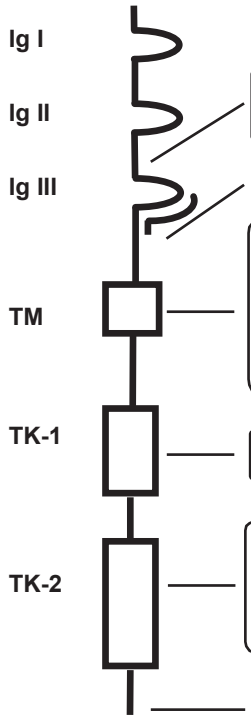
The patient characteristics and the histopathological data corresponding to the novel and the concurrent FGFR3 mutations in bladder carcinomas. The cases with the numbers 143, 172, 287 and 265 contained two concurrent FGFR3 mutations in one tumor. The cases 287, 213, 17 and 55 presented with the novel mutations reported here for the first time.

Case #	Age/sex	Stage/grade*	Exon	nt Position**	Mutation	Predicted effect**
143	77 / M	pTa/G2	7 7	nt742 nt746	C→T C→G	R248C S249C
172	65 / M	pT3/G3	7 10	nt746 nt1114	C→G G→T	S249C G372C
287	68 / M	pTa/G1	7 10	nt746 nt1144	C→G G→A	S249C G382R
265	74 / M	pTa/G2	7 10	nt746 nt1178	C→G C→A	S249C A393E
213	74 / M	pTa/G2	15	nt1955	A→T	K652M
17	66 / M	pTa/G1	15	nt1955	A→T	K652M
55	63 / M	pTa/G1	15	nt1955	A→C	K652T

** Pathological status according to the TNM97 and the WHO classification guidelines.*
*** The nucleotide (nt) and codon numbering refer to the FGFR3b isoform.*

Figure 2 indicates the locations and the nature of the missense mutations associated with skeletal disorders and bladder carcinomas. The frequencies of the various *FGFR3* mutations in bladder cancer are also indicated. These numbers are based on the previously published series by Cappellen et al.[5], Billerey et al.[16], van Rhijn et al.[17] and Sibley et al.[18] (n=273 patients) and the series described in this paper (n=297 patients).

Figure 2



FGFR3	MUTATION	SKELETAL DYSPLASIAS (GERMLINE)	BLADDER CANCER (SOMATIC)
Ig II	R248C	TD I	24 (8.1%)
Ig II	S249C	TD I	214 (71.8%)
Ig II	P250R	MC	0 (0%)
Ig III	N328I*	HCH	0 (0%)
TM	G370/372C	TD I	16 (5.4%)
TM	S371/373C	TD I	0 (0%)
TM	Y373/375C	TD I	30 (10.1%)
TM	G375/377C	ACH	0 (0%)
TM	G380/382R	ACH	1 (0.3%)
TM	A391/393E	C+AN	5 (1.7%)
TK-1	I538/540V**	HCH	0 (0%)
TK-1	N540/542K,T,S**	HCH	0 (0%)
TK-2	K650/652E	TD II	4 (1.3%)
TK-2	K650/652M	SADDAN	2 (0.7%)
TK-2	K650/652Q	HCH	1 (0.3%)
TK-2	K650/652N	HCH	0 (0%)
TK-2	K650/652T	?	1 (0.3%)
	X807/809C,G,L,R,W	TD I	0 (0%)
			n = 298 (100%)

FGFR3 mutations associated with skeletal disorders and bladder carcinomas. A schematic diagram of the structure of FGFR3 is shown (IgG I-III, immunoglobulin-like domains; TM, transmembrane domain; TK-1 and -2, tyrosine kinase domains). The locations of the missense mutations associated with skeletal dysplasias (TDI, thanatophoric dysplasia type I; TDII, thanatophoric dysplasia type II; MC, Muenke craniosynostosis; HCH, hypochondroplasia; ACH, achondroplasia; C+AN, Crouzon syndrome with acanthosis nigricans; SADDAN, severe achondroplasia with developmental defect and acanthosis nigricans) and bladder carcinomas are indicated. The positions are numbered according to the FGFR3c/FGFR3b numbering. The number of occurrences and the percentage of each FGFR3 mutation in mutated bladder tumors are given. These data were obtained from 570 bladder tumors of which 273 were reported before[5,16-18] and 297 were reported here. The different mutations found in skeletal dysplasias have been obtained from the review of Passos-Bueno et al.[6] and the work of Bellus et al.[11] and Winterpacht et al.[27].

**This region of FGFR3b corresponding to a region of FGFR3c where a mutation associated with hypochondroplasia (N328I) was previously found was only examined in 26 of the 570 bladder tumors studied so far.[5]*

***This region containing mutations associated with hypochondroplasia was only examined in 89 of the 570 bladder tumors studied so far.[5,18]*

DISCUSSION

FGFR3 mutations occur frequently in bladder carcinoma.[5,16-18] The vast majority of somatic *FGFR3* mutations identified in bladder cancer are identical to those found in thanatophoric dysplasia.[7,9] In the previous published series, only two *FGFR3* mutations (A391/393E and K650/652Q) did not correspond to thanatophoric dysplasia mutations.[17,18] However, both mutations have been found to be associated with milder types of skeletal dysplasia: the A391/393E mutation with the Crouzon syndrome with acanthosis nigricans and the K650/652Q mutation with hypochondroplasia. In this new series of 297 bladder carcinomas reported here, we identified 181 *FGFR3* mutations. Ninety-six percent (173/181) of these somatic mutations have been previously reported to be associated with thanatophoric dysplasia. However, other activating *FGFR3* mutations including the three described here for the first time in bladder cancer (G380/382R, K650/652M and K650/652T), have never been reported to be associated with thanatophoric dysplasia.

The activating G380/382R mutation, which affects the transmembrane domain, is responsible for almost all (~97%) cases of achondroplasia.[20,22] Achondroplasia is the most common form of non-lethal skeletal dysplasia, affecting approximately 1 in 15,000 to 1 in 40,000 live births.[7] This mutation is here reported in cancer for the first time.

The activating K650/652M mutation, located in the kinase domain, has already been found in SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans)[23] and in a primary multiple myeloma[12]. Transient transfection studies have demonstrated that the K650/652M mutation results in stronger constitutive activation of FGFR3 than does the K650/652E mutation responsible for thanatophoric dysplasia.[23] Nevertheless, despite multiple physical and neurological impairments, most SADDAN patients survive past infancy without the need for prolonged ventilator support.[7,23]

The K650/652T mutation has never been reported before in either skeletal dysplasia or cancer. However, other mutations of the same codon are associated with either thanatophoric dysplasia (K650/652E), SADDAN (K650/652M) or hypochondroplasia (K650/652N and K650/652Q).[10,11,23] Bellus et al.[11] investigated all the possible amino acid substitutions resulting from single nucleotide changes in the 650/652 codon and showed that the K650/652T mutation leads to constitutive activation of the FGFR3 tyrosine kinase. This activation was equivalent to that observed with the K650/652N and K650/652Q (hypochondroplasia) mutations but was considerably weaker than observed with the K650/652E and K650/652M mutations associated with thanatophoric dysplasia and SADDAN, respectively.

The identification of mutations in bladder cancers identical (K650/652Q)[18] or similar (K650/652T) (this study) to mutations found in hypochondroplasia suggests that other hypochondroplasia mutations, such as the I538/540V and N540/542K,T,S mutations in exon 13 may also occur in this carcinoma.[6,24] In

other words, possible hypochondroplasia mutations in other exons than 15 may have escaped their detection in bladder cancer. Among the 570 bladder tumors studied so far, only 89 have been studied for the hypochondroplasia mutations located in exon 13.[5,18] No activating mutations were detected in this exon for the 89 cases. Nevertheless, the percentage of mutations not corresponding to thanatophoric dysplasia in bladder cancer might be slightly higher than suggested.

Another novel finding presented here was the occurrence of two simultaneous *FGFR3* mutations in 4 tumors (case-numbers: 143, 172, 265 and 287, Table 1). The PCR-SSCP analysis of exon 7 in the case with number 404 provided a strong indication that the two concurrent mutations (R248C and S249C), are carried by the two different alleles (not shown). For the remaining 3 cases, this remains to be determined because these tumors had no loss of heterozygosity at the *FGFR3* locus (not shown) and RNA was not available.

From a clinical perspective, the presence of *FGFR3* mutations in bladder carcinomas, identical to the mutations found in patients with non-lethal skeletal disorders (hypochondroplasia, achondroplasia, SADDAN and Crouzon syndrome) suggests that the patients with these FGFR3-related syndromes might have a higher risk to develop bladder cancer than the general population. However, to our knowledge, there are no reports on a higher incidence of cancer in these patients, so it is possible that the non-lethal *FGFR3* mutations do not sufficiently activate the protein to an extent that is needed for tumor formation or other genes must be affected for bladder tumor formation. On the other hand, the predisposition may have gone unnoticed to clinicians, especially if we consider the relative low prevalence of non-lethal skeletal syndromes (~10.000 cases in the USA). For example, the retinoblastoma gene, cloned 18 years ago, has been demonstrated to play a role in many carcinomas, including bladder carcinomas. Yet, it was only recently proven that hereditary retinoblastoma patients have a higher risk to develop lung cancer than unaffected individuals.[25]

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PART IV

General Discussion

13

Summary & Future Perspectives
Samenvatting (Dutch)
Sommaire (French)

SUMMARY

Urothelial cell carcinoma (UCC) of the urinary bladder is a serious health problem. The disease accounts for 3.2% of all new cancer cases worldwide. UCC of the bladder is the 5th most common cancer and the 9th leading cause of cancer death. In most patients (± 75%), UCC is superficial (i.e. pTa-pT1) at first presentation. There are two problems in the management of these patients. First, superficial UCC of the bladder is characterized by a high risk of recurrence (55-85%), with most recurrences in the first year after trans-urethral resection (TUR). These recurrences are detected by urethro-cystoscopy (UCS), the gold standard, which is performed every 3-4 months, often in combination with urine cytology (UC) as an adjunct. After a two-year recurrence free period, the intensity of follow up may be lowered. UCS and UC are routinely used in the daily clinical practice. However, UCS is invasive, bothersome for the patients and costly. Unfortunately, UC is characterized by a low sensitivity, especially for low grade UCC. Hence, there is room for improvement, i.e. a need for development of techniques to diagnose superficial bladder cancer recurrences in urine and/or to identify a subset of patients who require a less frequent follow up. The second concern in managing patients with UCC is to prevent progression (10-15%) to muscle invasive, potentially lethal UCC. The ability to distinguish superficial tumors with invasive capabilities from those unlikely to become invasive would be of great clinical benefit. Carcinoma in situ (CIS), a flat and high grade (G3) manifestation of superficial UCC is especially associated with an unfavorable disease course. However, individual prediction of prognosis by clinical and pathological features is still very difficult. Superficial UCC that maintains a more malignant phenotype might be better treated with radical cystectomy in an early stage of the disease. This explains the need for more accurate predictors of UCC-progression. With the recent advances in molecular biology, research is more and more focusing on understanding the molecular mechanisms associated with bladder carcinogenesis. Therefore, molecular markers may aid in solving the two UCC management problems outlined above. If molecular markers prove to have additional value over the established clinico-pathological variables, they could become part of the clinical practice. This thesis deals with these two problems concerning the management of patients with UCC. Molecular markers for diagnosis in urine and prognosis of UCC were analyzed in various clinical settings.

Part one gives a short introduction on UCC in general perspective and contains the scope of this thesis (**chapter 1**).

In the second part of the thesis, we evaluated Microsatellite analysis (MA); a test based on PCR reactions with DNA of the urine sediment, in different clinical settings. **Chapter 2** provides an overview of the accuracy of the current urine markers to diagnose recurrent UCC. The sensitivity (patients with a recurrent lesion and positive urine test divided by all patients with recurrent UCC) and specificity (patients without recurrence and a negative test divided by all

patients without recurrent UCC) were determined for 18 urine markers. All the urine markers reviewed had a higher sensitivity, but a lower specificity than conventional cytology for recurrent UCC. The sensitivities increased with tumor grade. In this meta-analysis confined to studies on recurrent disease, MA had a sensitivity of 82% and a specificity of 89% for detection of UCC recurrences. We found a considerably lower sensitivity to detect recurrent UCC for 13/18 urine markers in comparison with earlier review articles where no distinction between primary and recurrent UCC was made. The reason for this reduced sensitivity is most likely a higher percentage of small, pTa and low grade UCC among recurrent lesions compared to primary tumors. At this time, sufficient clinical evidence to change the cystoscopic follow-up scheme with the use of a urine marker is not available. **Chapter 3** describes a phase II study on urinary MA, which we performed in a cross-section of 109 patients who visited the outpatient department for a follow-up UCS. Earlier investigations in other clinical settings and with a limited number of patients showed that MA had a high potential to diagnose UCC in urine. Our study was conducted to investigate whether MA was also capable of diagnosing UCC in patients who were under surveillance after TUR. MA detected 18/24 recurrent tumors. The undetected UCCs were small pTaG1 lesions. In 5 of 9 cases where no tumor was seen at UCS but MA was positive, an UCC was found within 6 months after MA. In the same group of patients, MA was more sensitive (74%) than the BTAstat test (56%) or conventional urine cytology (22%). A drawback of MA was the exclusion of 7 patients due to an insufficient amount of DNA in the urine sediment and another 9 patients with leukocyte abundance. Nevertheless, MA appeared a reliable molecular test in urine for detection of recurrent UCC, sometimes even before cystoscopical evidence of the disease. **Chapter 4** describes MA in conjunction with the *Fibroblast Growth Factor Receptor 3 (FGFR3)* mutation, a genetic marker for favorable UCC. This study was initiated to explore the possibility to improve the sensitivity of MA by adding *FGFR3* mutation analysis to the molecular marker panel. The tests were carried out on tumor- and urine samples of 59 UCC patients allowing a direct comparison of molecular changes in tumor and urine. Conventional cytology was used as a reference and a full clinical record of each patient was obtained. UCCs with a higher stage or grade had more microsatellite alterations per tumor. Conversely, *FGFR3* mutations were only found in pTaG1-2 lesions. The sensitivity of MA in *FGFR3* positive and negative UCC was 71% and 91%, respectively. When a Microsatellite alteration was detected in tumor tissue, the chance to find the same alteration in the urine was 35% regardless of *FGFR3* status. The chance to find the same *FGFR3* mutation in the urine was 52%. Additional alterations in the urine that were not present in the UCC were only seldom reported (1.8%). By combining the *FGFR3* mutation with MA, the sensitivity of molecular cytology could be enhanced to 89% without compromising specificity. The similarity of the molecular profiles in urine and tumor corroborate the clonal relation of tumor cells in the urine and the

original tumor(s). In **chapter 5**, the value of MA for patients under surveillance after radiotherapy was investigated. The follow up of these patients is more or less the same as for the patients with superficial UCC. However, UCS and UC are often difficult to interpret in case of previous radiotherapy. Therefore, MA was tested in this particular patient group. Although only 6 recurrences were found in the 49 patients, MA detected 5 of them. The specificity values for routine urine cytology, MA and expert urine cytology were 85%, 93% and 97%, respectively. In addition, the negative predictive value of MA (true negative MA / all negative MA) was 99% (70/71 urine samples) for this group. The latter is especially interesting because the positive predictive value of UCS (40% in this series) is much lower for irradiated patients. The results of this study indicate that MA may also be a useful tool for surveillance of patients who were previously treated by radiotherapy.

Part three of this thesis focuses on the prognostic value of the *Fibroblast Growth Factor Receptor 3 (FGFR3)* mutation in UCC. Its relation to favorable disease will be discussed as well as its relation to clinical variables and other molecular markers. **Chapter 6** gives an overview of the clinico-pathological and molecular prognostic features in (superficial) UCC. The last part of this chapter gives a short introduction of the *FGFR3* gene. The **chapters 7 & 8** contain the first two papers published on the favorable nature of the *FGFR3* mutation in UCC. The *FGFR3* mutation was detected in 70/98 (71%) of pTaG1-2 UCC. Conversely, invasive G3 lesions had a mutation in only 7% of tumors. Moreover, the mutation was absent in 20 cases of CIS, again pointing to the favorable nature of the *FGFR3* mutation in UCC. White blood cells and normal-looking urothelial biopsies of patients with a *FGFR3* mutation in their bladder tumor lacked *FGFR3* mutations. This emphasized the somatic nature and the tumor specificity of these mutations in UCC. The *FGFR3* mutation was also associated with a low recurrence rate of superficial UCC in the fist year after a TUR. In 57 patients with superficial UCC, only 6% of UCS was positive for patients with a *FGFR3* mutation. This was 28% for patients who had no *FGFR3* mutation in their tumor. These two preliminary studies showed that *FGFR3* is the first gene to be mutated at a high frequency in favorable (pTa G1-2) UCC. This mutation is the first genetic marker to selectively identify a large group of patients with favorable disease characteristics. Furthermore, the results presented in these two chapters suggest that the frequency of UCS can be reduced considerably for patients with *FGFR3* positive tumors. In **chapter 9** the results of a large, retrospective multi-center study are displayed. The purpose of this study was to compare the *FGFR3* mutations to other molecular markers and to obtain long term clinical follow up. In addition, the reproducibility of pathological grade and molecular variables was determined. In this study of 286 patients with primary UCC, the *FGFR3* mutation was related to the expression of three immunohistochemical markers (MIB-1, P53 and $P27^{kip1}$) known to be associated with prognosis. *FGFR3* mutations were detected in 172/286 (60%) of UCCs. G1-tumors had a

FGFR3 mutation in 88% of cases, G3-tumors in 16%. Conversely, aberrant expression patterns of MIB-1, P53 and $P27^{kip1}$ were seen in 5%, 2% and 3% of G1-tumors and in 85%, 60% and 56% of G3-tumors, respectively. In multivariate analysis with recurrence rate, progression and disease specific survival as endpoints, the combination of *FGFR3* and MIB-1 proved of independent significance for all 3 disease parameters. With regard to disease progression of superficial UCC, the difference in the clinical course between UCC combining *FGFR3* mutation / MIB-1 normal (N=138) and those combining *FGFR3* wild type / MIB-1 high (N=47) was evident. Interestingly, the 61 remaining patients with tumors characterized by absence of *FGFR3* mutation and normal MIB-1 expression or by a *FGFR3* mutation and high MIB-1 expression behaved more or less the same representing an intermediate prognosis. These results led to the proposal of a molecular grading model for UCC distinguishing three molecular grades (mG1-3): mG1 (favorable prognosis), mG2 (intermediate prognosis) and mG3 (poor prognosis). Moreover, the molecular Grade was more reproducible than pathological grade (91% vs. 47–61%). Based on the observations in the chapters 7, 8 and 9, it was concluded that the *FGFR3* mutation represents the favorable molecular pathway of papillary UCC. Furthermore, molecular Grading provides a new, simple and highly reproducible tool to determine UCC prognosis. **Chapter 10** shows that *FGFR3* mutations are also common in urothelial papilloma, a rare urothelial neoplasm. Papillomas are generally considered as a separate diagnostic entity because of their benign natural behavior. Therefore, they are currently classified apart from papillary lesions of low malignant potential (PUN-LMP, G1) and low grade papillary urothelial carcinomas (LG-PUC, G1-2) by the 1998 World Health Organization / International Society of Urological Pathology consensus classification. In our study, *FGFR3* mutations were found in 9/12 papillomas and in 68/79 pTaG1 lesions (reclassified as PUN-LMP and LG-PUC in 62 and 17 cases, respectively). Besides urothelial papillomas, the *FGFR3* mutation analysis was also performed for 21 ovarian Brenner tumors. This tumor was chosen for its resemblance to low grade UCC from a cytological point of view. However, no *FGFR3* mutations were found in Brenner tumors. This report was the first to describe a genetic defect in urothelial papilloma and we suggested from a molecular perspective, that urothelial papillomas should be classified together with all well differentiated urothelial neoplasms. **Chapter 11** describes the distribution of *FGFR3* mutations and P53 over-expression (assumed if expression level >10%) for 260 patients in UCC. As described in the chapters 7 and 8, the *FGFR3* gene is mutated in a high percentage of low grade UCCs. Therefore, the purpose of this study was to evaluate the relationship between *FGFR3* mutations and over-expression of the P53 tumor suppressor protein as a manifestation of a mutation in the *P53* gene. The over-expression of P53 and/or the mutant *P53* gene have been reported in earlier studies as an unfavorable marker in UCC. *FGFR3* mutations and P53 over-expression were observed in 59% and 25% of tumors, respectively. Together, the *FGFR3* and

P53 over-expression described 79% of UCC. However, alterations in *FGFR3* and P53 coincided in only 5.7% of UCC. Because each of these alterations was also associated with opposite clinico-pathological parameters, it was proposed that mutations in *FGFR3* and *P53* mark two alternative genetic pathways in papillary UCC pathogenesis. **Chapter 12** gives an overview of the distribution of *FGFR3* mutations detected in UCC and the corresponding germline mutations, which are responsible for a wide variety of skeletal anomalies. Almost all *FGFR3* mutations in earlier studies corresponded to the mutations that lead to thanatophoric dysplasia, a congenital disorder that is generally lethal shortly after birth. This study reported a few new *FGFR3* mutations in UCC that matched non-lethal skeletal syndromes. Therefore, patients with skeletal syndromes associated with *FGFR3* mutations and a relative normal life span (for example: achondroplasia) might be at a higher risk for development of bladder tumors than the general population provided that the *FGFR3* mutation on its own is capable of bladder tumor formation.

FUTURE PERSPECTIVES

The focus of this thesis was twofold. Nowadays, UCC recurrences are detected by urethro-cystoscopy (UCS) with urine cytology (UC) as an adjunct. These techniques are used routinely but they are not ideal. UCS is invasive, bothersome and costly and UC lacks sensitivity. Hence, there is room for improvement to detect superficial bladder cancer recurrences in urine. In the studies that were performed with Microsatellite analysis (MA) of urine as a non-invasive diagnostic tool, we found that MA was highly sensitive and equal in specificity to urine cytology and capable of competing with UCS for recurrent UCC diagnosis. In a recent review (chapter 2), MA was highly sensitive and specific for patients under surveillance in comparison with other urine markers. Since MA is still a time consuming and labor intensive technique, future studies should address the automation and upscaling of the assay. Moreover, multi-center studies, in which the results have to be delivered in a certain time frame, will be essential if this assay is to become a commonly enjoyed clinical tool. Furthermore, one of the next steps for MA may include a randomized study, in which half of the patients will receive a less stringent cystoscopical follow up scheme in combination with MA. Such a study, which is coordinated by the Erasmus MC, is ongoing at this time. Only via this way the question whether MA is the urine test that can be used to reschedule UCS can be answered. The patients with G3-UCC should be excluded from such a protocol since the possible drawbacks of MA have not been fully investigated yet and delay in the diagnosis of recurrent disease of these patients with a high risk of progression may not occur. Additionally, it should be anticipated that the sensitivity of MA might decrease if the G3 patients will be excluded. So far, much attention has been paid to the performance (sensitivity and specificity) of urine markers. Studies on the opinion of the patients have received little attention. Vriesema et al. found that 89% of the patients will prefer UCS if the sensitivity of the urine marker is less than 90%.[1] This prerequisite can not be satisfied by any of the currently available tests in urine. Probably, these patients did not realize that a sensitivity of >90% will also be a problem for UCS, as this gold standard is at best 90% sensitive and specific.[2] On the other hand, Schrag et al. analyzed the adherence to surveillance among patients with superficial UCC in a 3 year follow up period and they reported that only 40% of patients had all the cystoscopical examinations, which were recommended by the clinical guidelines.[3] The opinion of patients should ideally be addressed with the use of validated questionnaires in a randomized study that may be performed simultaneously with a urine marker study (see above for example). Cost-effectiveness is becoming more important in the daily clinical practice. The current follow-up schemes largely contribute to the fact that the total costs per patient are the highest for UCC compared to other malignancies.[4] Lotan and Roehrborn found that a modified care protocol, in which UCS was alternated by a urine marker was cost-effective at a cost of less than $264 per test.[5] They also concluded that such a protocol warrants a

prospective, randomized evaluation to determine whether the benefits outweigh any potential drawbacks. Close monitoring of patients is essential for the detection of recurrent disease, which in turn requires repeated resection to rule out progression to invasive cancer. However, how close our surveillance should be, whether a urine marker may be of use to plan UCS and other aspects like patient opinion and costs are all issues that will have to be addressed in future prospective randomized evaluations. The high sensitivity and specificity of MA highlight its potential application in these studies. On the other hand, UCS is also a subject of study. Fluorescence cystoscopy with the intravesical application of photodynamic agents proved more sensitive than the conventional white light cystoscopy for the detection of UCC.[6] Moreover, a single fluorescent trans-urethral resection (TUR) was superior to white light TUR with respect to both residual tumor rate and recurrence free survival.[7] As MA of urine was highly sensitive and specific in studies with white light UCS as a reference, it would be interesting to analyze MA in combination with fluorescent UCS. Furthermore, the application of MA in other settings, like in screening protocols or as adjunct to UCS in high grade UCC, may be studied.

The second focus of this thesis was the prognostically favorable *FGFR3* mutation in UCC. The association of this mutation with low stage and low grade UCC is evident and confirmed by other groups.[8,9] The suggestion made in this thesis that patients with a *FGFR3* mutation in their UCC require a less stringent follow up has to be confirmed in a prospective multi-center study. Perhaps, this can be performed in conjunction with the randomized study on MA. Progression to invasive UCC occurs in 10-15% of patients with superficial UCC. To identify those patients who will progress is a major challenge, especially because patients with progressive disease seem to have a worse prognosis than patients with primary invasive disease.[10] Unfortunately, predictability of progressive disease with the conventional clinical and pathological parameters is not sufficiently accurate in individual patients and it may only be used to identify (risk) groups of patients with UCC.[11,12] Because molecular grade, which depends on *FGFR3* and MIB-1 status was a significant predictor for recurrence rate, progression and disease specific survival and because molecular grade was more reproducible than pathological grade, there is a need for a prospective evaluation of this molecular grading system (see chapter 9). If such trials would come out positive and if *FGFR3* mutation analysis has additional value over the classical clinical and pathological parameters, then implementation of a *FGFR3* mutation assay for clinical purpose may happen. However, such studies take many years to perform. Other techniques like cDNA expression microarrays and proteomics might be especially useful for the individual prediction of prognosis as they analyze many molecular variables at the same time, thereby providing a more tailor-made risk profile. Dyrskjot et al. developed a 32-gene molecular classifier in an independent test set of 68 UCCs.[13] The classifier provided new predictive information in pTa tumors with regard to progression and recurrence

rate.[13] The authors are currently evaluating the classifier in a three-year prospective clinical study.[13] With the help of protein profiles, Vlahou et al. were able to detect 7 differentially expressed protein cluster regions that demonstrated differences between UCC and controls.[14] Here again, it is important that the initial results of the cDNA expression microarray and proteomics investigations are confirmed by other research groups and that prospective trials are required before considering implementation of these techniques.

The bladder is an easy accessible organ for instillation therapy. Intravesical treatments with immunotherapeutic (BCG) and chemotherapeutic agents have been developed, tested extensively in many study-protocols and they are widely used in the daily clinical practice to reduce recurrences and prevent progression. Two promising clinical studies showed that a combination of mitomycin-C and local hyperthermia was more effective than standard endovesical chemotherapy and that this combination had promising value in intermediate and high risk superficial bladder cancer compared to the literature data on BCG and intravesical chemotherapy.[15,16] Hanel et al. found in an experimental orthotopic rat model that the intravesical application of reovirus (an oncolytic virus that selectively destroys cancer cells with an activated Ras pathway) was safe and more effective with regard to tumor-free survival than BCG or normal saline.[17] FGFR3 is part of a family of 4 tyrosine kinase receptors. In the physiological situation, various signal transduction pathways are initiated via FGF binding to the receptor. A mutation in *FGFR3* causes constitutive activation of the receptor. Considering the results of Hanel et al.,[17] it is of interest that *FGFR3* and *RAS* mutations in multiple myeloma both lead to the activation of the same downstream pathway.[18] Because the involvement of FGFR3 in UCC is a recent discovery, an attempt to develop drugs to target this receptor has not yet been undertaken. The incidence of superficial UCC and the high percentage of *FGFR3* mutations in these tumors together with the high percentage of over-expression of FGFR3 in UCC[19] justify further study in this direction. The clinical success of kinase inhibitors in other types of cancer underlines this.[20] For superficial UCC, a possible intravesical application for FGFR3 inhibition would be preferable and such a study would be of great interest.

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SAMENVATTING (DUTCH)

Het urotheelcelcarcinoom (UCC) van de urineblaas is een serieus gezondheidsprobleem. Wereldwijd is 3.2% van alle nieuwe kankerdiagnosen aan deze ziekte toe te schrijven. UCC is de 5e meest voorkomende vorm van kanker en de 9e meest voorkomende vorm van kanker gerelateerde sterfte. De meeste patiënten (± 75%) presenteren zich met oppervlakkig UCC (pTa-pT1). Er zijn twee dilemma's bij het begeleiden van deze patiënten. Als eerste kenmerkt het oppervlakkig UCC zich door een hoge kans op één of meerdere recidieven (55-85%), waarbij de meeste recidieven gevonden worden in het eerste jaar na een transurethrale resectie (TUR). Deze recidieven worden gedetecteerd door middel van urethro-cystoscopie (UCS), de gouden standaard, die elke 3-4 maanden na een TUR wordt verricht, vaak in combinatie met urine cytologie (UC) als hulpmiddel. Na een recidief vrije periode van twee jaar kan de frequentie van de controles verlaagd worden. UCS en UC worden in de dagelijkse praktijk routinematig toegepast. UCS is echter invasief, vervelend voor de patiënten en duur. UC wordt helaas gekenmerkt door een lage sensitiviteit, met name voor laaggradig UCC. Er is derhalve ruimte voor verbetering, dat wil zeggen de ontwikkeling van methoden om oppervlakkige blaaskanker recidieven in urine te diagnosticeren en/of een subgroep van patiënten aan te wijzen die minder vaak gecontroleerd zouden kunnen worden. Een tweede punt van zorg bij het begeleiden van patiënten met UCC is het voorkomen van progressie (10-15%) naar spier-invasief carcinoom waaraan de patiënt mogelijk kan overlijden. De mogelijkheid om onderscheid te maken tussen oppervlakkige tumoren met en zonder invasieve capaciteiten zou klinisch van grote importantie zijn. Carcinoma in situ (CIS), een vlakke, hooggradige (G3) variant van oppervlakkig UCC is met name geassocieerd met een slecht ziekteverloop. Ondanks alles is het voorspellen van prognose voor een individuele patiënt met behulp van klinische en pathologische variabelen zeer moeilijk. Oppervlakkig UCC van een meer kwaadaardig phenotype kan wellicht in een vroeg stadium van de ziekte beter behandeld worden door middel van een radicale cystectomie. Dit verklaart waarom betere voorspellers voor UCC progressie nodig zijn. Met de recente vooruitgang in de moleculaire biologie, is ook de research steeds meer gericht op het begrijpen van de moleculaire mechanismen die ten grondslag liggen aan blaascarcinogenese. Moleculaire markers zouden kunnen helpen bij het oplossen van de hierboven beschreven dilemma's. Als deze markers additionele waarde blijken te hebben ten opzichte van de huidige klinische en pathologische parameters, dan zouden ze onderdeel kunnen worden van de dagelijkse klinische praktijk. Dit proefschrift gaat over deze twee dilemma's t.a.v. de zorg voor patiënten met UCC. Moleculaire markers voor diagnose in urine en prognose van UCC werden geanalyseerd in verschillende klinische kaders.

Deel één bevat een korte introductie over UCC in het algemeen en beschrijft de opzet van dit proefschrift (**hoofdstuk 1**).

In het tweede gedeelte van het proefschrift evalueerden we in verschillende klinische kaders de Microsatelliet analyse (MA); een test gebaseerd op PCR reacties met DNA uit het urinesediment. **Hoofdstuk 2** geeft een overzicht van de waarde van de huidige urine markers voor de diagnose van een recidief UCC. De sensitiviteit (patiënten met een recidief en een positieve urine test gedeeld door alle patiënten met een recidief UCC) en specificiteit (patiënten zonder recidief en een negatieve test gedeeld door alle patiënten zonder recidief UCC) werden bepaald voor 18 urine markers. Alle urine markers in het overzicht hadden een hogere sensitiviteit maar een lagere specificiteit voor recidief UCC dan de conventionele cytologie. De sensitiviteit nam toe met tumorgraad. In deze meta-analyse die alleen studies over recidief ziekte bevatte, was de sensitiviteit van de MA 82% en de specificiteit 89%. We vonden een behoorlijk lagere sensitiviteit t.a.v. recidief UCC voor 13/18 urine markers in vergelijk met eerdere overzichtsartikelen waarbij geen onderscheid gemaakt is tussen primair en recidief UCC. De reden voor deze lagere sensitiviteit is waarschijnlijk een hoger percentage klein, pTa en laaggradig UCC bij recidief laesies t.o.v. primaire tumoren. Vooralsnog ontbreekt voldoende klinisch bewijs om het cystoscopische controle schema te wijzigen m.b.v. een urine marker. **Hoofdstuk 3** bevat een fase II studie over MA van urine die uitgevoerd werd in een dwarsdoorsnede van 109 patiënten die de polikliniek bezochten voor een controle UCS. Eerdere studies uitgevoerd in andere klinische kaders en met een lager aantal patiënten hadden reeds laten zien dat MA veelbelovend was om UCC in urine te diagnosticeren. Deze studie werd uitgevoerd om te onderzoeken of MA ook in staat was UCC te diagnosticeren bij patiënten die onder controle waren na een TUR. MA detecteerde 18/24 recidieven. De niet gevonden recidieven waren kleine pTaG1 laesies. In 5 van de 9 gevallen waarbij geen tumor werd gezien bij UCS maar waarbij MA wel positief was, werd toch nog een UCC gevonden binnen 6 maanden na MA. Bij dezelfde patiënten bleek MA sensitiever (74%) dan de BTAstat test (56%) en conventionele UC (22%). Nadelen van de MA waren de exclusie van 7 patiënten vanwege een onvoldoende hoeveelheid DNA in het urinesediment en 9 patiënten vanwege teveel leukocyten in de urine. Desondanks bleek MA een betrouwbare moleculaire test in urine om een recidief UCC te vinden, soms zelfs voordat er bij cystoscopie een recidief gezien werd. **Hoofdstuk 4** gaat over MA in combinatie met de *Fibroblast Groei Factor Receptor 3 (FGFR3)* mutatie, een genetische marker voor gunstig UCC. Deze studie werd uitgevoerd om te onderzoeken of het mogelijk was de sensitiviteit van de MA te verhogen door de *FGFR3* mutatie analyse eraan toe te voegen. De tests werden uitgevoerd op tumor- en urine monsters van 59 UCC patiënten waardoor er een directe vergelijking van de moleculaire veranderingen in tumor en urine mogelijk was. Conventionele cytologie werd gebruikt als referentie en een volledig klinisch rapport was beschikbaar van iedere patiënt. De UCCs met een hoger stadium en graad hadden meer microsatelliet veranderingen per tumor. Daarentegen werden

FGFR3 mutaties alleen gevonden bij pTaG1-2 laesies. De sensitiviteit van de MA alleen was voor *FGFR3* positief en negatief UCC respectievelijk 71% en 91%. Wanneer een microsatelliet verandering in tumor werd aangetoond, dan was de kans om dezelfde verandering ook in urine te vinden 35%. Dit was onafhankelijk van de *FGFR3* status. De kans om dezelfde *FGFR3* mutatie in urine te vinden was 52%. Veranderingen in de urine die niet in de corresponderende tumor gevonden werden, waren zeldzaam (1.8%). Door het combineren van de *FGFR3* mutatie met de MA kon de sensitiviteit van moleculaire cytologie verbeterd worden tot 89% zonder daling van de specificiteit. De overeenkomst tussen de moleculaire profielen van urine en tumor in deze studie onderstrepen de clonale relatie van tumor cellen in de urine en de originele tumor(en). De waarde van de MA voor patiënten die gecontroleerd worden na bestraald te zijn op hun blaas is onderzocht in **hoofdstuk 5**. De controle van deze patiënten verloopt min of meer op dezelfde wijze als bij patiënten met oppervlakkig UCC. UCS en UC zijn echter vaak moeilijk te interpreteren na radiotherapie. Om die reden werd deze bijzondere groep patiënten getest m.b.v. MA. Hoewel er slechts 6 recidieven gevonden werden bij 49 patiënten, ontdekte de MA er 5 van. De specificiteit van routine UC, MA en expert UC was respectievelijk 85%, 93% en 97%. Daarnaast was de negatief voorspellende waarde van MA (echt negatieve MA / alle negatieve MA uitslagen) 99% (70/71 urine monsters) bij deze groep. Dit laatste is met name interessant omdat de positief voorspellende waarde van de UCS (40% in deze serie) veel lager is na bestraling. De resultaten van deze studie laten zien dat de MA ook van waarde kan zijn bij patiënten die gecontroleerd worden na bestraling.

Het derde deel van dit proefschrift gaat over de prognostische waarde van de *FGFR3* mutatie bij UCC. De relatie van deze mutatie met een gunstig ziektebeloop en de relatie tot andere klinische variabelen en moleculaire markers werd bediscussieerd. **Hoofdstuk 6** bevat een overzicht van de klinische, pathologische en moleculaire kenmerken van het (oppervlakkig) UCC. Het laatste gedeelte van dit hoofdstuk geeft een korte inleiding over het *FGFR3* gen. De **hoofdstukken 7 & 8** bevatten de eerste twee publicaties over het gunstige karakter van de *FGFR3* mutatie bij het UCC. Een *FGFR3* mutatie werd gevonden bij 70/98 (71%) van de pTaG1-2 UCC. Aan de andere kant had slechts 7% van de invasieve G3 tumoren zo'n mutatie. Verder was de mutatie afwezig in 20 gevallen van CIS wat ook weer wijst op het gunstige karakter van de *FGFR3* mutatie bij het UCC. Witte bloedcellen en biopten van normaal urotheel bij patiënten met een *FGFR3* mutatie in hun tumor lieten geen *FGFR3* mutaties zien. Dit benadrukte het somatische karakter en de tumorspecificiteit van deze mutaties voor UCC. De *FGFR3* mutatie was tevens geassocieerd met een lage recidief-frequentie van oppervlakkig UCC in het eerste jaar na een TUR. Bij 57 patiënten met oppervlakkig UCC, was slechts 6% van de UCS positief voor de patiënten met een *FGFR3* mutatie. Dit was 28% voor de patiënten die geen *FGFR3* mutatie in hun tumor hadden. Deze twee preliminaire studies maakten

duidelijk dat *FGFR3* het eerste gen is dat vaak gemuteerd is bij gunstig (pTaG1-2) UCC. Daarnaast suggereren de resultaten uit deze twee hoofdstukken dat de frequentie van UCS behoorlijk verlaagd zou kunnen worden voor de patiënten met *FGFR3* positieve tumoren. In **hoofdstuk 9** worden de resultaten van een grote, retrospectieve, multicentrische studie getoond. Het doel van deze studie was het vergelijken van de *FGFR3* mutatie met andere moleculaire markers en het verkrijgen van een lange klinische follow-up. Tevens werd de reproduceerbaarheid van de pathologische graad en de moleculaire variabelen bepaald. De *FGFR3* mutatie werd in deze studie van 286 patiënten met primair UCC vergeleken met de expressie van drie immunohistochemische markers (MIB-1, P53 en $P27^{kip1}$) waarvan het bekend is dat ze correleren met prognose. Een *FGFR3* mutatie werd gevonden bij 172/286 (60%) van de UCCs. G1 tumoren hadden een mutatie in 88% van de gevallen, G3 tumoren in 16%. Daarentegen werden aberrante expressie niveaus van MIB-1, P53 en $P27^{kip1}$ gevonden bij respectievelijk 5%, 2% en 3% van de G1 tumoren en 85%, 60% en 56% van de G3 tumoren. De combinatie van *FGFR3* en MIB-1 was significant in de multivariaat analyse met als eindpunten recidief frequentie, progressie en ziekte vrije overleving. Het verschil in klinisch beloop met betrekking tot progressie van oppervlakkig UCC was evident t.a.v. de combinaties *FGFR3* mutatie / MIB-1 normaal (N=138) en *FGFR3* wild type / MIB-1 verhoogd (N=47). Het was interessant dat de overige 61 patiënten met tumoren zonder *FGFR3* mutatie en normale MIB-1 expressie of tumoren met een *FGFR3* mutatie en tevens een hoge MIB-1 expressie, een nagenoeg identiek klinisch beloop hadden dat tussen de twee eerdere groepen lag en zich kenmerkte door een redelijke prognose. Dit resultaat stelde ons in staat een moleculair graderingsmodel voor UCC te propageren waarin 3 moleculaire graden te onderscheiden zijn (mG1-3): mG1 (goede prognose), mG2 (redelijke prognose) en mG3 (slechte prognose). Daarnaast was de moleculaire Graad reproduceerbaarder dan de pathologische graad (91% vs. 47-61%). Op basis van de hoofdstukken 7, 8 en 9 was het de conclusie dat de *FGFR3* mutatie selectief de gunstige moleculaire “pathway” van papillair UCC karakteriseert. Verder is moleculair graderen een nieuw, eenvoudig en zeer reproduceerbaar instrument om de prognose van UCC te bepalen. **Hoofdstuk 10** laat zien dat *FGFR3* mutaties ook frequent voorkomen bij het papilloom wat een zeldzame afwijking van het urotheel is. Papillomen worden in het algemeen beschouwd als een aparte diagnostische entiteit vanwege hun benigne natuurlijk beloop. Daarom zijn ze ook in de 1998 World Health Organization / International Society of Urological Pathology consensus classificatie als zodanig geclassificeerd naast de papillaire urotheel-laesies met lage maligniteits potentie (PUN-LMP, G1) en de laaggradige papillaire urotheel carcinomen (LG-PUC, G1-2). In onze studie werden *FGFR3* mutaties gevonden bij 9/12 papillomen en bij 68/79 pTaG1 laesies (na review geclassificeerd als PUN-LMP en LG-PUC in respectievelijk 62 en 17 gevallen). Naast papillomen van het urotheel werd de *FGFR3* mutatie

analyse ook verricht op 21 Brenner tumoren van het ovarium. Deze tumor werd gekozen vanwege de gelijkenis met laaggradig UCC vanuit cytologisch oogpunt. Desalniettemin werden er geen *FGFR3* mutaties gevonden bij Brenner tumoren. Deze studie beschreef als eerste een genetisch defect bij papillomen van het urotheel. Verder suggereerden we dat vanuit een moleculair perspectief, de papillomen van het urotheel samen in één groep met alle andere goed gedifferentieerde tumoren van het urotheel geclassificeerd zouden moeten worden. **Hoofdstuk 11** beschrijft de verdeling van *FGFR3* mutaties en P53 over-expressie (als expressie niveau >10%) voor 260 patiënten met UCC. Zoals beschreven in de hoofdstukken 7 en 8, is het *FGFR3* gen gemuteerd bij een hoog percentage van laaggradige UCCs. Het doel van deze studie was derhalve om de relatie tussen *FGFR3* mutaties en over-expressie van het P53 tumor suppressor eiwit als uiting van een mutatie in het *P53* gen te evalueren. De over-expressie van P53 en/of het mutant *P53* gen zijn in eerdere studies beschreven als ongunstige markers m.b.t. het UCC. *FGFR3* mutaties en P53 over-expressie zijn bij respectievelijk 59% en 25% van de tumoren gevonden. Samen beschrijven de *FGFR3* mutatie en de P53 over-expressie 79% van de UCCs. Echter, veranderingen in *FGFR3* en P53 kwamen slechts bij 5.7% van de UCCs tegelijkertijd voor. Omdat deze genetische veranderingen ook nog eens geassocieerd waren met tegenovergestelde klinische en pathologische parameters, opperden wij dat mutaties in *FGFR3* en *P53* twee verschillende genetische “pathways” in de pathogenese van UCC markeren. **Hoofdstuk 12** geeft een overzicht van de verdeling van de *FGFR3* mutaties die gevonden zijn in UCC en dezelfde mutaties in de kiembaan die verantwoordelijk zijn voor een grote verscheidenheid aan skelet afwijkingen. Bijna alle *FGFR3* mutaties in eerdere studies m.b.t. UCC kwamen overeen met de mutaties die thanatophore dysplasie veroorzaken, een aangeboren afwijking die in het algemeen snel na de geboorte tot de dood leidt. Deze studie rapporteert enkele nieuwe *FGFR3* mutaties bij UCC die gevonden zijn bij niet-lethale syndromen met skeletafwijkingen. Onder de voorwaarde dat de *FGFR3* mutatie op zich voldoende is voor het aanmaken van een blaastumor, zou het dus wel eens zo kunnen zijn dat patiënten met een *FGFR3* geassocieerde skeletafwijking en een relatief normale levensverwachting (bijvoorbeeld: Achondroplasie) een hogere kans hebben op het krijgen van blaastumoren in vergelijk met de algehele populatie.

SOMMAIRE (FRENCH)

Le carcinome cellulaire de l'épithélium (CCU) vésical est un problème de santé grave. Au niveau mondial 3,2% de tous les nouveaux diagnostics de cancer sont imputés à cette maladie. Le CCU est le cancer qui représente en fréquence le cinquième des plus répandus et le neuvième des formes mortelles. Le plus grand nombre des patients (75%) se présentent avec un carcinome superficiel (pTa-pT1). Le suivi de ces patients pose deux dilemmes. Premièrement, il y a un grand risque que ce CCU superficiel récidive dans 55 à 85% des cas. La plupart de ces récidives sont retrouvées dans la première année après une résection trans-uréthrale (RTU). Les récidives sont détectées au moyen d'uréthro-cystoscopie (UCS), l'étalon or, qui est appliquée tous les trois à quatre mois après une RTU, souvent en combinaison avec la cytologie urinaire (CU) comme moyen de support. Après une période de non-récidives de deux ans la fréquence des contrôles pourra être diminuée. UCS et CU font partie de la pratique journalière. Cependant, la UCS est invasive, désagréable pour le patient et coûteuse. Malheureusement le désavantage de la CU est la faible sensibilité notamment à un CCU de basse gradation. Une amélioration est donc possible i.e. le développement de méthodes pour identifier des récidives de tumeurs de vessie superficielles dans l'urine et/ou indiquer un sous-groupe de malades qui pourraient être contrôles moins souvent. Un deuxième point de souci dans le suivi des malades de CCU est de prévenir la progression, 10 à 15%, vers un carcinome invasif musculaire dont le malade pourrait mourir. La possibilité de distinguer les tumeurs superficielles avec et sans capacités invasives serait d'une grande importance au niveau de la clinique. Carcinome in situ (CIS), une variante plane de haut grade(G3) du CCU superficiel est notamment associé à une mauvaise progression de la maladie. Faire des pronostics pour le malade individuel à l'aide de variables cliniques et pathologiques est malgré tout très difficile. Dans in stade précoce de la maladie le CCU superficiel d'un phénotype plus malin pourra probablement être mieux traité par une systectomie radicale. Cela explique pourquoi il faut de meilleurs pronostics de la progression du CCU. Par l'évolution récente dans la biologie moléculaire les recherches sont de plus en plus orientées vers les compréhensions des mécanismes moléculaires qui sont à la base de la genèse du cancer de la vessie. Des marqueurs moléculaires pourraient aider à résoudre les deux dilemmes décrits ci-dessus. Si ces marqueurs se trouvent avoir des valeurs additionnelles vis-à-vis les paramètres cliniques et pathologiques actuels ils pourraient faire partie de la pratique clinique quotidienne. Cette thèse traite ces deux dilemmes concernant le suivi des malades de CCU. Des marqueurs moléculaires pour le diagnostic dans l'urine et le pronostic du CCU ont été analysés dans des cadres cliniques différents.

La première partie contient une courte introduction sur le CCU en général et décrit le plan de cette thèse (**chapitre 1**).

Dans la deuxième partie de cette thèse nous avons évalué l'analyse Microsatellite (AM) dans des cadres cliniques différents. L'AM est un test basé sur PCR avec ADN pris du sédiment urinaire. **Chapitre 2** donne un aperçu de la valeur des marqueurs d'urine actuels pour le diagnostic d'un CCU récidivant. La sensibilité (des patients avec une récidive et un test d'urine positif divisés par tous les patients avec un CCU récidivant) et la spécificité (des patients sans récidive et un test négatif divisés par tous les patients sans récidive de CCU) était définie pour 18 marqueurs d'urine. Tous les marqueurs d'urine dans l'aperçu avaient, en ce qui concerne la récidive du CCU un degré de sensibilité plus haut, mais une spécificité plus basse que la cytologie conventionnelle. La sensibilité augmentait avec le degré de la tumeur. Dans cette méta-analyse qui ne contenait que des études de récidives la sensibilité de l'AM était de 82% et la spécificité de 89%. Nous avons trouvé une sensibilité nettement plus basse par rapport à la récidive du CCU. Pour 13 des 18 marqueurs d'urine en comparaison avec des aperçus précédents où la distinction entre un CCU primaire et une récidive n'était pas faite. La raison de cette faible sensibilité est probablement un plus élevé pourcentage de petit pTa et un CCU de bas grade dans des lésions récidives par rapport à des tumeurs primitives. Pour le moment nous n'avons pas encore suffisamment de preuves cliniques pour changer le schéma de contrôle cystoscopique à l'aide d'un marqueur d'urine. **Chapitre 3** contient une étude phase II sur l'AM d'urine réalisée sur une coupe transversale de 109 patients venus à la policlinique pour un contrôle de UCS. Des études antérieures faites dans d'autres cadres cliniques et avec un nombre plus restreint de patients avaient montré que l'AM était très prometteuse pour diagnostiquer le CCU dans l'urine. Cette étude était faite pour examiner si avec l'AM il était possible de diagnostiquer le CCU chez des patients qui avaient subi un RTU. L'AM a détecté 18 sur 24 récidives. Les récidives non-trouvées étaient de petites lésions pTaG1. Dans 5 cas sur 9 où l'on n'a pas vu de tumeur dans la UCS mais où par contre l'AM était positive, on a quand-même trouvé un CCU avant 6 mois après l'AM. Chez ces mêmes patients l'AM s'est avérée plus sensitive (74%) que le test BTAstat (56%) et la conventionnelle CU (22%). Les désavantages de l'AM était l'exclusion de 7 patients à cause d'une quantité insuffisante de ADN dans le sédiment de l'urine et 9 patients à cause de trop de leucocytes dans l'urine. Pourtant, l'AM s'est avérée un test moléculaire dans l'urine fiable pour trouver un CCU récidivant, parfois même avant qu'on ait vu une récidive par cystoscopie. **Chapitre 4** traite l'AM en combinaison avec la mutation de *FGFR3* («*Fibroblast Growth Factor Receptor 3*»), un marqueur génétique pour un CCU positif. Cette étude a été entreprise pour voir s'il était possible de hausser la sensibilité de l'AM en y ajoutant l'analyse de la mutation de *FGFR3*. Les tests étaient effectués sur des prises de tumeurs te d'urine de 59 patients de CCU ce qui a permis une comparaison directe des mutations moléculaires dans la tumeur et l'urine. La cytologie conventionnelle était utilisée comme système de référence. On pouvait disposer d'un dossier clinique complet de chaque patient. Les CCUs plus

avancés avaient plus de mutations microsatellites par tumeur. Les mutations de *FGFR3* par contre n'étaient trouvées qu'avec les lésions pTaG1-2. La sensibilité de l'AM seule était pour *FGFR3* positive et négative CCU, 71% et 91% respectivement. Quand une mutation microsatellite était détectée dans la tumeur, la chance de trouver la même mutation dans l'urine était de 35%, ceci indépendamment du dossier médical de *FGFR3*. La chance de trouver la même mutation de *FGFR3* dans l'urine était de 52%. Rares étaient les mutations dans l'urine qui n'étaient pas trouvées dans les tumeurs correspondantes (1,8%). En combinant la mutation de *FGFR3* avec l'AM on a pu améliorer la sensibilité de la cytologie moléculaire jusqu`à 89% sans que la spécificité diminue. La similitude des profiles moléculaires de l'urine et de la tumeur soulignent dans cette étude la relation clonale de cellules carcinogènes dans l'urine et les tumeurs primitives. **Chapitre 5** évalue la valeur de l'AM pour les patients dont la vessie a été traités aux rayons et qui sont examinés après par UCS. Le protocole du suivi de ces patients est plus ou moins pareil à celui des patients d'un CCU superficiel. Pourtant le UCS et la CU sont souvent difficiles à interpréter après radiothérapie. C'est pour cette raison que ce groupe de patients exceptionnel a été testé à l'aide de l'AM. Quoique 6 récidives seulement soient trouvées chez 49 patients, l'AM en a détecté 5. Les spécificités de routine de CU, d'AM et de CU expert étaient de 85%, 93% et 97% respectivement. En plus, la valeur prédicative négative de l'AM (AM vraiment négative / tous les résultats négatifs de l'AM) était de 99% (70/71 prises d'urine) chez ce groupe. Ceci est d'autant plus intéressant que la valeur prédicative positive du UCS (40% dans cette série) est beaucoup plus basse après radiothérapie. Les résultats de cette étude montrent que l'AM prend de la valeur aussi pour les patients qui sont examinés après un traitement par rayons.

La troisième partie de cette thèse traite la valeur prognostique de la mutation de *FGFR3* dans le CCU. La relation entre cette mutation et un bon pronostic et la relation de celle-là avec d'autres variables et marqueurs moléculaires ont été discutées. **Chapitre 6** présente un aperçu de tous les caractères cliniques, pathologiques et moléculaires du CCU superficiel. La dernière partie de ce chapitre donne une courte introduction au gène *FGFR3*. Les **chapitres 7 et 8** contiennent les deux premières publications sur le caractère positif de la mutation de *FGFR3* dans le CCU. Une mutation de *FGFR3* était trouvée dans 70/98 (71%) des CCU de pTaG1-2. De l'autre côté, il n'y avait que 7% des tumeurs invasives et G3 qui montraient une telle mutation. La mutation était absente dans 20 cas des CIS ce qui indique aussi le caractère positif de la mutation de *FGFR3* dans la CCU. Des leucocytes et des biopsies urothéliques normales des patients avec une mutation de *FGFR3* dans leur tumeur ne montraient pas de mutations de *FGFR3*. Ceci souligne le caractère somatique et la spécificité tumorale de ces mutations dans le CCU. La mutation de *FGFR3* était en même temps associée à une faible fréquence de récidive de CCU superficiel dans la première année après une RTU. Chez 57 patients avec

un CCU superficiel seulement 6% des UCS étaient positives pour les patients avec une mutation de *FGFR3*. Cela constituait 28% pour les patients qui n'avaient pas de mutation de *FGFR3* dans leur tumeur. Ces deux études préliminaires montraient que le *FGFR3* est le premier gène qui a souvent muté dans un CCU favorable (pTaG1-2). Les résultats de ces deux chapitres suggèrent en plus que la fréquence des UCS pourrait être considérablement réduite pour des patients avec des tumeurs positives pour *FGFR3*. **Chapitre 9** montre les résultats d'une grande étude rétrospective et multicentrée. Par cette étude nous avons voulu comparer la mutation de *FGFR3* avec d'autres marqueurs moléculaires et obtenir un long «follow-up» clinique. Nous avons déterminé en même temps la reproductibilité du grade pathologique et les variables moléculaires. Dans cette étude de 286 patients avec un CCU primaire, la mutation de *FGFR3* a été comparée avec l'expression de trois marqueurs immunohistochimiques (MIB-1, P53 et $P27^{kip1}$) dont il est connu qu' ils corrèlent avec le pronostic. Une mutation était trouvée dans 172/286 (60%) des CCU. Les tumeurs de G1 avaient une mutation dans 88% des cas, les tumeurs de G3 en avaient dans 16%. Par contre, des niveaux d'expression aberrants de MIB-1, P53 et $P27^{kip1}$ étaient trouvés dans respectivement 5%, 2% et 3% des tumeurs de G1 et dans 85%, 60% et 56% des tumeurs de G3. La combinaison de *FGFR3* et MIB-1 était significatif dans l'analyse multivariate avec comme point finals la fréquence de récidives, la progression et la survie par guérison totale. La différence du cours clinique entre la progression du CCU superficiel et la combinaison de la mutation de *FGFR3* / MIB-1 normal (N=138) et *FGFR3* «wild type» / MIB-1 élevé (N= 47) était évidente. Il était intéressant que les autres 61 patients avec des tumeurs sans mutation de *FGFR3* et une expression normale de MIB-1 ou des tumeurs avec une mutation de *FGFR3* et une expression de MIB-1 élevée avaient un cours clinique identique, situé entre les deux groupes ci-devant et caractérisé par un pronostic raisonnable. Ce résultat nous a permis de propager un modèle moléculaire de gradation pour le CCU où trois grades moléculaires sont à distinguer (mG1-3): mG1 (pronostic favorable), mG2 (pronostic raisonnable) et mG3 (pronostic défavorable). En plus, le grade moléculaire était plus reproductible que le grade pathologique (91% vs. 47-61%). Les chapitres 7,8 et 9 ont mené à la conclusion que la mutation de *FGFR3* caractérise d'une façon sélective le «pathway» moléculaire favorable du CCU papillaire. En plus, la gradation moléculaire est un nouvel outil, simple et très reproductible pour déterminer le pronostic de CCU. **Chapitre 10** montre que les mutations de *FGFR3* se produisent aussi fréquemment dans le papillome, une malformation rare de l'urothélium. Les papillomes sont généralement considérés comme une entité diagnostique à part à cause de leurs cours naturels bénins. C'est pourquoi qu'ils ont été classifiés comme tels dans le consensus de 1998 de l'Organisation Mondiale de la Santé / «International Society of Urological Pathology», à côté des lésions de l'urothélium papillaire avec un bas potentiel de malignité (PUN-LMP, G1) et les carcinomes papillaires de l'urothéliun grade bas

(LG-PUC, G1-2). Dans notre étude des mutations de *FGFR3* étaient trouvées dans 9/12 papillomes et dans 68/79 lésions pTaG1 (après une révision classifiées comme PUN-LMP et LG-PUC dans 62 et 17 cas respectivement). À côté des papillomes de l'urothélium l'analyse de la mutation de *FGFR3* a été faite aussi sur 21 tumeurs Brenner de l'ovaire. Cette tumeur était choisie à cause de la ressemblance avec le CCU de bas grade du point de vue cytologique. Pourtant, aucune mutation de *FGFR3* n'était trouvée dans les tumeurs Brenner. Cette étude était la première à décrire un défaut génétique dans des papillomes de l'urothélium. Nous avons aussi suggéré que du point de vue moléculaire les papillomes devraient être classifiés dans un seul groupe avec toutes les autres tumeurs bien différenciées de l'urothélium. **Chapitre 11** décrit la répartition des mutations de *FGFR3* et la sur-expression P53 (niveau d'expression >10%) pour 260 patients avec un CCU. Comme c'est décrit dans les chapitres 7 et 8 le gène *FGFR3* a muté dans un pourcentage élevé des CCUs de bas grade. Le but de cette étude était par conséquent d'évaluer la relation entre les mutations de *FGFR3* et la sur-expression de la protéine P53, comme manifestation d'une mutation dans le gène *P53*. La sur-expression de P53 et/ou le gène mutant ont été décrits dans des études antérieures comme des marqueurs défavorables par rapport au CCU. Des mutations de *FGFR3* et des sur-expressions P53 ont été trouvées dans respectivement 59% et 25% des tumeurs. Les mutations et les sur-expressions ensemble répertorient 79% des CCUs. Cependant, ce n'était que dans 5,7% des CCUs qu'il y avait simultanément les deux changements. Comme ces mutations génétiques étaient en plus associées à des paramètres cliniques et pathologiques contraires nous avons suggéré que des mutations dans *FGFR3* et *P53* marquent deux «pathways» génétiques différents dans la pathogenèse du CCU. **Chapitre 12** fait passer en revue la division des mutations de *FGFR3* trouvées dans les CCUs et les mêmes mutations germinales qui sont responsables d'une grande variété de malformations du squelette. Presque toutes les mutations de *FGFR3* dans des études antérieures concernant le CCU correspondaient avec les mutations responsable de nanisme thanatophore, une anomalie congénitale qui conduit généralement à la mort tout de suite après la naissance. Cette étude signale quelques nouvelles mutations de *FGFR3* dans le CCU trouvées dans des syndromes avec des anomalies du squelette non-létales. À condition que la mutation de *FGFR3* en soi suffise pour développer une tumeur de vessie, il pourrait bien être possible que les patients avec une anomalie du squelette associée au *FGFR3* et une espérance de vie relativement normale (p.ex. achondroplasie) courent un plus grand risque que des tumeurs de vessie soient détectées chez eux en comparaison avec l'ensemble de la population.

PART V

Appendices

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Curriculum Vitae

Bas van Rhijn was born on April 5th 1969 in Aalsmeer, The Netherlands. After graduating from college at the Augustinianum College in Eindhoven, he started Medical School at the Catholic University of Nijmegen in 1988. In 1996 he graduated and started a two-year period of pre-residency at the Academic Hospital of Nijmegen. During this pre-residency he conducted research at the department of Urology and wrote his first scientific publication on the prognostic value of high-risk Quanticyt. At the same department, he also completed an optional internship (Prof.dr. F.M.J. Debruyne). In July 1998 he obtained his medical degree.

As of October 1998 until December 2001 he worked on the present dissertation at the departments of Pathology and Urology of the Erasmus MC, Rotterdam. The thesis project was accepted as AGIKO project (i.e. a resident in clinical research) by the Registration Council of Medical Specialists (MSRC) in 2000.

During this predominantly scientific period, he worked as a clinical trial doctor in the treatment of patients with metastatic prostate and renal cell carcinoma in co-operation with the department of Clinical Oncology, Erasmus MC, Rotterdam.

In 2001, he received the Vlietstra Award of the Dutch Urological Association (DUA) for his presentation at the DUA-congress on the favorable disease course of *FGFR3* mutations in bladder cancer.

In 2002 he started his residency for his specialty training in Urology. From January 2002 until December 2003 he worked at the department of General Surgery at the 'Reinier de Graaf Gasthuis' in Delft (Dr. L.P.S. Stassen) and as of January 2004 he returned to the Erasmus MC, Rotterdam, department of Urology to continue his Urological training (Prof.dr. J.L.H.R. Bosch, Prof.dr. C.H. Bangma, Dr. G.R. Dohle).

In 2004, he received the Prof.dr. W.A. Moonen Award of the DUA for his research on molecular aspects of bladder cancer.

On January 18, 2003 he married Sandra van Hoorn.

Curriculum Vitae
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Bas van Rhijn is geboren op 5 April 1969 in Aalsmeer. Na het behalen van zijn Gymnasium-B diploma aan de Augustinianum Scholengemeenschap te Eindhoven, begon hij in 1988 aan de opleiding Geneeskunde aan de Katholieke Universiteit Nijmegen. In 1996 behaalde hij zijn doctoraal en begon hij aan zijn co-schappen in het Academische Ziekenhuis Nijmegen. Gedurende deze co-schappen deed hij onderzoek bij de afdeling Urologie en schreef zijn eerste wetenschappelijke publicatie over prognostische factoren bij een hoog risico Quanticyt uitslag. Bij dezelfde afdeling deed hij een keuze co-schap (hoofd: Prof.dr. F.M.J. Debruyne). In juli 1998 behaalde hij zijn artsexamen.

Van oktober 1998 tot december 2001 werkte hij aan deze dissertatie bij de afdelingen Pathologie en Urologie van het Erasmus MC te Rotterdam. Registratie als AGIKO (assistent geneeskundige in opleiding tot klinisch onderzoeker) door de MSRC (Medische Specialisten Registratie Commissie) volgde in 2000.

Tijdens deze voornamelijk wetenschappelijke periode was hij tevens werkzaam als klinische trial-arts bij de behandeling van patiënten met gemetastaseerd prostaat- en niercelcarcinoom in samenwerking met de afdeling Klinische Oncologie van het Erasmus MC.

Begin 2001 ontving hij de Vlietstra Prijs van de Nederlandse Vereniging voor Urologie (NVU) voor zijn voordracht over het gunstige ziektebeloop van patiënten met een *FGFR3* mutatie in hun blaastumor.

In 2002 startte hij met het klinische gedeelte van de opleiding tot uroloog. Van januari 2002 tot december 2003 werkte hij in het kader van de vooropleiding bij de afdeling Algemene Heelkunde van het Reinier de Graaf Gasthuis in Delft (opleider: Dr. L.P.S. Stassen). Sinds januari 2004 is hij teruggekeerd naar het Erasmus MC, afdeling Urologie (opleiders: Prof.dr. J.L.H.R. Bosch, Prof.dr. C.H. Bangma, Dr. G.R. Dohle).

Eind 2004 ontving hij de Prof.dr. W.A. Moonen Prijs van de NVU voor zijn onderzoek naar moleculaire aspecten van blaaskanker.

Op 18 januari 2003 trouwde hij met Sandra van Hoorn.

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Bas van Rhijn, 2005