Molecular Markers in the Development, Diagnosis and Progression of Non-muscle Invasive Bladder Cancer

Willemien Beukers

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Molecular Markers in the Development, Diagnosis and Progression of Non-muscle Invasive Bladder Cancer

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PART I

GENERAL INTRODUCTION
AND SCOPE OF THE THESIS

Chapter 1

General Introduction and scope of the thesis

EPIDEMIOLOGY

In 2008 there were approximately 386,300 new cases of bladder cancer (BC) worldwide.[1] The highest incidence rates are found in Europe and the United States. BC is more predominant in males with a male:female ratio of 4:1. The incidence rates increase with age, with a peak incidence in the sixth decade of life. Under the age of 40, BC is highly uncommon with reported incidence rates varying between 1-2%.[2-4] BC is a multifactorial disease, with smoking and occupational exposure as major risk factors in the developed countries. In less developed countries, mostly Africa and the Middle East, the major cause is chronic infection with Schistosoma hematobium.[1, 5] Bladder tumors related to schistosomiasis are mostly from the squamous cell type, whereas 90% of the bladder tumors in the developed countries are from the urothelial cell type. In this thesis we will only discuss bladder cancer from the urothelial type.

CLINICAL PRESENTATION AND DIAGNOSIS

The major symptom in patients presenting with bladder cancer is painless microscopic or macroscopic hematuria, i.e. blood in the urine. Patients presenting with hematuria form a large part of the urological population. Yet, only 5-20% of the hematuria is caused by BC. Therefore, hematuria is not a specific symptom and could be caused by various conditions, such as kidney stones, urinary tract infections, benign prostate hyperplasia or kidney diseases. Furthermore, not all patients with bladder cancer will present with hematuria. Other symptoms are dysuria, frequency, recurrent bladder infections or symptoms of obstruction. The gold standard for the detection of BC is white light cystoscopy (WLC). Cystoscopy is an endoscopic procedure as depicted in figure 1.1. The urologist places a rigid or flexible scope through the urethra into the bladder. The flexible glass fibers in the scope transfer the image from the tip of the scope to a

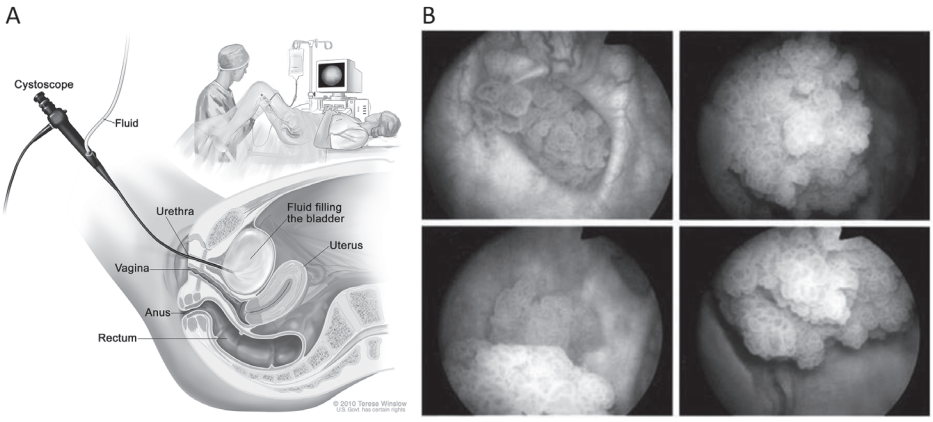

Figure 1.1 *(A) Investigation of the bladder by cystoscopy. (B) Bladder tumor visualized by white light cystoscopy. Source: www.stfranciscare.org and www.urology.ie*

screen. By using the cystoscope the urologist is able to examine the entire bladder wall. Yet, the sensitivity for the detection of BC is not optimal ranging from 46% to 80%.[6-8] Especially flat malignant lesions (Carcinoma In Situ) are difficult to visualize and to distinguish from inflammatory lesions and as a consequence some of these high-grade tumors are missed. To improve the diagnostic accuracy new endoscopic techniques are developed. Fluorescence or photodynamic cystoscopy (PDD) uses photoactive agents, e.g. 5-aminolevulinic acid (5-ALA) or hexaminolevulinate (HAL), which preferentially accumulates in tumor cells. Under blue light with a wavelength of 375-440 nm, the cells with the accumulated agent emit a red fluorescence, while normal bladder tissue remains blue.[9] Sensitivity for PDD ranges from 76%-97% and is superior to WLC, especially for the detection of CIS. Yet, high false-positives rates are observed particularly after recent TUR, BCG treatment or urinary tract infections.[8] Another newly developed technique is narrow band imaging (NBI). This technique enhances the contrast between mucosal and microvascular tissue by using a light source consisting of two different wavelengths (425 and 540nm). This light only penetrates the superficial mucosal layer and is strongly absorbed by hemoglobin. The contrast between normal mucosa and capillaries helps to distinguish between normal urothelium and more vascularized malignant areas. NBI improves de detection of BC over WLC, with reported sensitivities of 85%-100%.[10-13] However, comparable with PDD, high false-positives rates are observed.

Next to cystoscopy, urine cytology is performed. Urine cytology microscopically investigates urine for the presence of tumor cells. Urine generally contains normal urothelial cells shed from the urinary tract. A major limitation of urine cytology is its limited ability of detection low-grade (LG) tumor cells. A large meta-analysis by Lotan *et al* showed a median sensitivity for the detection of grade 1 tumors of 12%, 26% for grade 2 tumors and 64% for grade 3 tumors. Overall, the specificity is high (99%) both for low-grade and high-grade lesions.[14]

STAGING AND GRADING

BC is classified according to the widely used TNM-system approved by the Union for International Cancer Control (UICC).[15] The T-stage indicates the extent of local tumor invasion (Fig 1.2, table 1.1). The N-stages specify the spread of malignant cells to regional lymph nodes and the M-stage describes whether there are distant metastasis (Table 1.1). There are two main groups of bladder cancer: the non-muscle invasive bladder tumors (NMIBC) and muscle-invasive bladder tumors (MIBC). Ta-tumors, together with T1-tumors form the group of NMIBC. These tumors are limited to the urothelial cell layer (Ta) or grow into the lamina propria (T1). Carcinoma in situ (CIS), a flat tumor limited to the urothelial cell layer, is also considered as NMIBC. Despite its superficial growth character, CIS has a high malignant potential. The MIBCs comprise stage T2-, T3- and T4-tumors. These tumors invade the detrusor muscle (T2), the perivesical fat (T3) or grow into surrounding organs (T4). Most common sites of metastasis are lung, liver and bone.

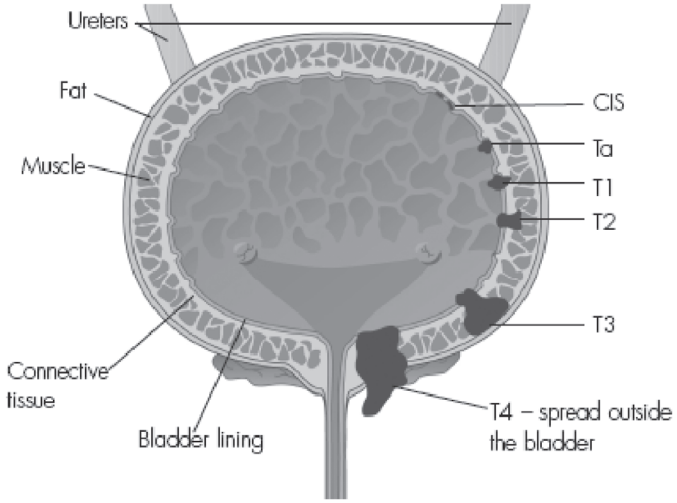

Figure 1.2 Different stages of bladder cancer. All urothelial cell carcinomas derive from the urothelial tissue layer called bladder lining. Source: www.cambridgeurologypartnership.co.uk

Table 1.1: 2009 TNM classification of urinary bladder cancer

T - Tumor		
Tx		Tumor cannot be assessed
T0		No evidence of primary Tumor
Ta		Non-invasive papillary carcinoma
Tis		Carcinoma in situ: 'flat Tumor'
T1		Tumor invades subepithelial connective tissue
T2		Tumor invades muscle
	T2a	Tumor invades superficial muscle (inner half)
	T2b	Tumor invades deep muscle (outer half)
T3		Tumor invades perivesical tissue
	T3a	Microscopically
	T3b	Macroscopically (extravesical mass)
T4		Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall
	T4a	Tumor invades prostate, uterus or vagina
	T4b	Tumor invades pelvic wall or abdominal wall
N - Lymph nodes		
Nx		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Metastasis in a single lymph node in the true pelvis
N2		Metastasis in multiple lymph nodes in the true pelvis
N3		Metastasis in a common iliac lymph node(s)
M - Distant metastasis		
M0		No distant metastasis
M1		Distant metastasis

Source: TNM Classification of Malignant Tumours, 7th Edition, 2009.

BC is graded by the WHO-grading system (table 1.2). Currently, two systems are used: the 1973 WHO-grading system and the 2004 WHO-grading system (Fig 1.3). In the 1973 WHO-classification urothelial tumors are divided based on the degree of differentiation into four different categories: papilloma and grade 1 to 3. A major limitation of the 1973 WHO-grading system is the not well-defined criteria for the three different tumor grades, resulting in high percentages of tumors classified as grade 2. Furthermore, there is a great inter-observer variation.[16-18] Therefore, a new grading system was introduced in 1998 by the World Health Organization (WHO), which was finally adopted in 2004. It consists of papillary urothelial neoplasms of low malignant potential (PUNLMP), low-grade (LG) urothelial cancer, and high-grade (HG) urothelial cancer,[19] with detailed criteria for the different histologic conditions. Yet, the inter-observer variability is still high. Therefore, there is no consensus whether using the 1973 or 2004 WHO-system.[20-23] In this thesis both systems have been used.

Table 1.2: WHO grading 1973 and 2004

1973 WHO grading
Urothelial papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
2004 WHO grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

Source: EAU guidelines on non-muscle invasive bladder cancer, 2015

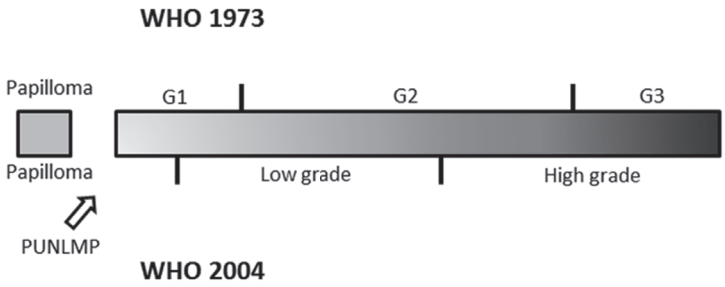

Figure 1.3 *Grading classification according the 1973 WHO system and the 2004 WHO system. Source: van Rhijn et al, Eur Urol, 2009*

TREATMENT

Transurethral resection (TUR) is the initial treatment for suspicious lesions and is performed to remove all visible tumor and to provide tissue for pathological assessment. The depth of tumor growth is of prognostic importance and defines the additional treatment. Hence, the presence of detrusor muscle in the TUR specimen is essential for accurate pathological evaluation. Furthermore, a radical TUR is crucial to achieve good prognosis.[24] A second TUR is recommended in case the initial resection is irradical or when the detrusor muscle is absent in the primary pathological specimen. Moreover, a re-TUR is advised in case a high grade T1 tumor has been detected at the initial TUR.[25] Pathological assessment of a re-TUR can result in a change of stage and further debulking of potential tumor cells has been shown to improve recurrence-free survival.[25-27] In addition to TUR, the EAU guidelines recommend a single dose of postoperative chemotherapy for patients with Ta/T1 disease in order to reduce the chance of developing recurrence.[28] Patients with non-muscle invasive disease with a high risk of recurrence or progression receive adjuvant instillations with Bacillus Calmette-Guérin (BCG) or chemotherapy in addition to the single postoperative dose. BCG invokes an immune response in the bladder which affects the cancer cells.[29] In case of BCG treatment failure cystectomy should be considered.[28]

The treatment of muscle invasive bladder cancer depends on the extent of invasion and the presence of metastasis. In the absence of metastasis, radical cystectomy is the optimal treatment, including removal of the bladder, adjacent organs and regional lymph nodes.[30] Neoadjuvant chemotherapy prior to cystectomy improves overall survival.[31] In case of metastatic disease, there are no curative options. Depending on patient's performance status systemic chemotherapy could be considered.

PROGNOSIS

Approximately 70% of bladder tumors are initially diagnosed as non-muscle invasive disease and 30% as muscle invasive disease. The overall prognosis of NMIBC patients is favorable with 5-year survival rates over 90%. Yet, the recurrence rate in patients with NMIBC is high: 70% of the patients will develop at least one recurrence within five years.[32] Currently, the recurrence and progression risk is calculated according to the EORTC risk tables (tables 1.3 and 1.4), including several clinical pathological factors: tumor size, multiplicity, prior recurrence rate, tumor stage, tumor grade, and the presence of concomitant CIS.[33] These EORTC risk scores were developed in 2006 based on results from 7 clinical trials. A limitation of this study was the low number of included patients treated with BCG. Nowadays, BCG is widely used as adjuvant intravesical therapy and it is known to reduce the risk of recurrence and progression.[34] A recent meta-analysis reported adjuvant BCG treatment as important prognostic factor for high-grade T1 patients.[35] Since the EORTC risk scores may not be accurate to predict prognosis in the BCG-treated patient, the CUETO risk score were added to the guidelines in 2015. These risk scores

were specifically designed for patients treated with BCG.[36] Recently, Vedder *et al* validated both risk scores on a large independent set of NMIBC patients and concluded that both risk scores still needed further improvement, especially for the prediction of recurrence risk.[37] Similarly, Xylinas *et al* investigated both risk scores in a large group of NMIBC and found that EORTC and CUETO risk scores lack discriminative ability for disease recurrence and progression.[38] Both risk scores are still highly dependent on the pathological review, which is subject to high interobserver and intraobserver variability. Molecular markers could be useful in the evaluation of tumor aggressiveness and support predicting prognosis. The search for new prognostic molecular markers is an ongoing investigation. Some of the promising prognostic markers are discussed in paragraph 1.7.1.

10-20% of the NMIBC will eventually progress to muscle invasive disease. Patients with muscle invasive have a much worse prognosis compared to patients with NMIBC with 5-year survival rates ranging between 30% and 60%.[30] Prognosis and treatment of MIBC is highly depending on tumor stage and grade. Patients with metastatic disease have a 5-year survival of only 10-15%.

Table 1.3: Weight scores for recurrence and progression risk

Factor	Recurrence	Progression
Number of tumors		
Single	0	0
2 to 7	3	3
≥8	6	3
Tumor size		
<3 cm	0	0
≥3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤1 rec/yr.	2	2
>1 rec/yr.	4	2
T category		
Ta	0	0
T1	1	4
CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total score	0–17	0–23

*CIS=Carcinoma in Situ. Source: Sylvester et al., Eur Urol **49:** 466, 2006*

Table 1.4: Probability of recurrence and progression according to total score

Recurrence score	Probability of recurrence 1 year (95% CI)	Probability of recurrence 5 years (95% CI)
0	15% (10%, 19%)	31% (24%, 37%)
1–4	24% (21%, 26%)	46% (42%, 49%)
5–9	38% (35%, 41%)	62% (58%, 65%)
10–17	61% (55%, 67%)	78% (73%, 84%)
Progression score	**Probability of progression 1 year (95% CI)**	**Probability of progression 5 years (95% CI)**
0	0.2% (0%, 0.7%)	0.8% (0%, 1.7%)
2–6	1.0% (.4%, 1.6%)	6% (5%, 8%)
7–13	5% (4%, 7%)	17% (14%, 20%)
14–23	17% (10%, 24%)	45% (35%, 55%)

*Source: Sylvester et al, Eur Urol **49:** 466, 2006*

SURVEILLANCE

The high recurrence rate in patients with NMIBC and the chance of developing progression requires an intensive and frequent follow-up. Current surveillance protocols are based on the EORTC risk calculator, which determines recurrence risk and progression (table 1.3 and 1.4).[33] Patients with tumors at low risk for recurrence and progression should have a cystoscopy at three months after initial TUR, according to the EAU guidelines.[28] If no tumor is found, the next cystoscopy is advised at nine months and yearly thereafter for a period of 5 years. Patients with high-risk tumors should have cystoscopy in combination with urine cytology after three months. If negative, cystoscopy and cytology should be repeated every three months for two years. After two years without a recurrence the follow-up should be continued with a cystoscopy every 6 months until 5 years and yearly thereafter. An in-between follow-up scheme is recommended to patients with intermediate risk-tumors (comprising approximately 30% of all patients) depending on personal and subjective factors. Patients with Tis should have the same follow-up schedule as high-risk patients, due to the high malignant potential of carcinoma in situ. All surveillance schedules begin from the start in case a recurrence is detected. This strict surveillance in combination with the high number of TURs makes BC one of the most expensive tumor types.[39, 40] Furthermore, cystoscopy is an invasive procedure causing pain and physical discomfort. Many research have been done developing non-invasive tests based on urinary markers in order to reduce costs and patient burden.[14, 41-43] Accuracy and reliability are the most important parameters in the development of a new urine test. Moreover, the ideal urinary marker is low in costs, easy to perform and highly reproducible. Many promising markers have been found, but large prospective validation studies and randomized controlled trials are lacking. Therefore, none of the newly investigated markers proved to be good enough to safely reduce or replace the number of cystoscopies. In paragraph 1.7.2. we discuss some of the potential urinary markers for BC surveillance.

MOLECULAR MARKERS IN BLADDER CANCER

Prognostic markers

Currently, recurrence and progression risk are calculated based on the EORTC risk table as discussed above. This risk calculator includes clinical and pathological variables to predict the chance of developing recurrent or progressive disease. Yet, high interobserver and intraobserver variability is observed in the pathological assessment. Molecular markers could improve prognostic accuracy in individual patients. Hence, the markers may be useful to identify patients with NMIBC, who will benefit from more invasive follow-up. Many research have been done in order to find the right marker for risk assessment and an extensive range of molecular alterations have been investigated to improve the prediction of prognosis. P53 is one of the most frequently investigated markers. Many studies revealed that *p53* mutation or p53 overexpression is related to disease progression.[44] Furthermore, *FGFR3* mutations are found in up to 70% of all NMIBC and several studies reported the positive association of *FGFR3* mutations on progression-free survival.[45-48] The proliferation marker Ki-67 is also related to progressive disease and therefore, van Rhijn *et al* combined the *FGFR3* mutation status and Ki-67 expression (assessed by staining with antibody MIB-1) into a new molecular grade. Molecular grade 1 (mG1) was defined as *FGFR3* mutant and low expression of MIB-1, mG2 comprised *FGFR3* wild-type tumors with low expression of MIB-1 or *FGFR3* mutant tumors with high expression of MIB-1 and mG3 included FGFR3 wild-type tumors with high expression of MIB-1.[47] The progression-free survival was significant better in patients with low molecular grade compared to the higher molecular grades. Furthermore, the molecular grades proved to be better reproducible than the original pathological grade. The addition of molecular grade to the EORTC risk scores resulted in an improvement of progression-risk prediction.[48] Dyrskjot *et al* developed a gene signature in order to predict progression in patients with NMIBC.[49] This gene signature was validated on an independent multicenter set of NMIBC patients. Patients with the progression signature showed a cumulative progression probability of 40% over time, compared to a cumulative progression probability of less than 15% for patients with a non-progression signature.[50] Sixty-six percent of the patients were correctly classified as progressors, with 66% specificity.

Aleman *et al* identified *PMF-1* hypermethylation in bladder cancer and showed the relation between loss of PMF1 expression and overall survival.[51] Alvarez-Mugica *et al* studied *PMF1* methylation and its association with clinical outcome in high-grade T1 BC patients treated with BCG.[52] Higher progression rates were observed in patients without methylation of *PMF1* compared to those with methylated *PMF1*. Futhermore, shorter disease-specific survival was observed in the group with unmethylated *PMF-1*. In contrast to the study by Aleman *et al,* methylation of *PMF1* appeared to have a favorable effect on clinical outcome in high-grade T1 BC patients treated with BCG. Methylation of *Myopodin* is also described as prognosticator in patients with high-grade T1 disease.[53] *Myopodin* methylation in bladder cancer was first described by Cebrian *et al* and was associated with increasing tumor stage and tumor grade.[54] Furtermore, there was a relation with poorer survival. Alvarez-Mugica *et al* investigated *Myo-*

podin methylation in patients with T1G3 disease and found significant shorter progression-free survival and disease-specific survival in patients with a *myopodin*-methylated tumor.[53]

Currently, no molecular markers are integrated in risk assessment or decision-making models in bladder cancer. The high heterogeneity within the different studies leads to irreproducible results and there is often lack of proper validation. Validation on large prospective multicenter patients series is needed before these prognostic markers can be introduced in the urological practice.

Urinary biomarkers

Considering the high frequency of cystoscopy for the follow-up of NMIBC, urinary markers would be extremely useful for the detection of BC, particularly for the detection of recurrent disease. Since the sensitivity of urine cytology for the detection of low-grade tumors is low, extensive research has been done in order to find new urinary biomarkers. Many markers have been found with sensitivity superior to urine cytology and currently, some of the newly developed tests are FDA-approved (e.g. Bladder Tumor Antigen, ImmunoCyt, Nuclear Matrix Protein-22, and Fluorescent In Situ Hybridization).[41, 55] Yet, none of the discovered markers have a significant role in bladder cancer surveillance, since marker performance is not high enough to safely reduce or replace the number of cystoscopies. Therefore, these urinary tests are only used in addition to cystoscopy. A major problem in studies investigating new markers is the selection of urine samples. Most studies included urine samples derived from patients with primary bladder tumors. The primary tumor is mostly larger in size and less well differentiated than recurrent tumors. Therefore, these tumors will shed more tumor cells in urine, resulting in high sensitivity. Subsequently, the marker sensitivity drops considerably when tested on urine samples derived from patients with a recurrent tumor.[43] Therefore, it is important to include follow-up urines in the search for novel surveillance markers. Furthermore, large longitudinal and randomized controlled trials are lacking. These studies are important to emphasize the possible anticipatory effect of urine analysis, meaning that urinary makers could detect BC before it is visible by cystoscopy. Moreover, the sensitivity of cystoscopy varies from 46% to 80%, which could lead to 'false-positive' urine results, since the tumor might not detected by cystoscopy.[6-8] Longitudinal analyses could anticipate on this. Zuiverloon *et al* investigated urine samples collected during follow-up from NMIBC patients with a *FGFR3* mutant primary tumor. Urine analysis with the *FGFR3* mutation assay revealed sensitivity for concomitant recurrence detection of 58%, with a positive predictive value (PPV) of 25%. The PPV increased when the time-interval was extended to 1 year: 55% of the positive urine samples were associated with a recurrence within a year and 81% of the positive urine samples were followed by a recurrence during the total follow-up period.[56] In the same line, van der Aa *et al* analyzed urine from NMIBC patients during follow-up with microsatellite analysis (MA) and showed a sensitivity for concomitant recurrence detection of 58%. Extending the follow-up period resulted in a 2-yr risk for recurrence detection of 83% in case the MA analysis was persistently positive.[57] Sanchez-Carbayo *et al* studied 106 patients under BC surveillance and collected their urine samples over a year. Samples were tested with three different urinary assays: urinary tumor bladder antigen (dectection of CK8, CK18),

CYFRA-21-1 (detection of CK19) and NMP22 (detection of nuclear matrix proteins). 31 patients recurred within a year and in 30 patients recurrence was identified by urine analysis before it was visible by cystoscopy. Recently, Schmidtz-Dräger *et al* published the WHO consensus on molecular markers in bladder cancer surveillance. The authors emphasized the importance of prospective analyses to define the consequences of a positive and negative test results before these markers could be implemented in clinical decision making.[58]

Molecular assays

The diagnostic and prognostic markers discussed in this thesis are summarized below.

Mutation analysis

Fibroblast Growth Factor Receptor 3 gene mutations

Fibroblast growth factor receptor 3 (FGFR3) belongs to a family of structurally related tyrosine kinase receptors encoded by four different genes. Activating mutations in the *FGFR3* gene are found in the germline of several autosomal dominant skeletal dysplasia syndromes, including hypochondroplasia, achondroplasia, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) and thanatophoric dysplasia types I and II.[59, 60] The major cellular defect in these FGFR3-related skeletal dysplasias is the impaired chondrocyte proliferation. Unlike its role in chondrocytes, FGFR3 activation results in a mitogenic effect in epithelial cells and these respond to the FGFR3 activation by increased proliferation by constitutive activation of the RAS-MAPK pathway (Figure 1.5). Remarkably, the majority of *FGFR3* mutations causing skeletal dysplasias also occur in cancer.[61] Cappellen *et al* were the first in describing *FGFR3* mutations in bladder cancer.[59] After this initial study, there were several studies that have identified 11 different *FGFR3* mutations in bladder cancer.[60] Extensive research has been done and demonstrated the association of *FGFR3* mutations with low tumor grade and stage. Approximately 70% of the non-muscle invasive bladder tumors harbor a mutation in the *FGFR3* gene compared to less than 20% of the muscle-invasive bladder tumors.[59, 62, 63] *FGFR3* mutant tumors have a more favorable prognosis and are less likely to progress.[45, 64, 65] Therefore, the *FGFR3* analysis could be used as prognostic marker.[46, 47, 63, 65] The absence of *FGFR3* mutations in normal urothelium allows the use of *FGFR3* mutation analysis as a tumor marker.[66] Previous studies demonstrated the ability of *FGFR3* mutation analysis for the detection of bladder cancer recurrences in voided urine (Figure 1.4).[56, 67, 68]

HRAS, KRAS, NRAS gene mutations

The oncogenes *HRAS*, *KRAS*, and *NRAS* are the founding members of a larger family of at least 35 related human proteins and belong to the class of small GTPases that function as an intermediary step in the RAS-MAPK signal transduction pathway. Activating mutations of the three *RAS* genes are frequently observed in different tumor types.[69] The activated *HRAS* gene was first identified in a bladder cancer cell line by Der *et al*.[70] Jebar *et al* studied *RAS* mutations in bladder tumors and found a mutation frequency of 13% in tumors of all stages and grades, most often *HRAS* and *KRAS*.[71] Similar to the *FGFR3* mutations, *RAS* mutations affect

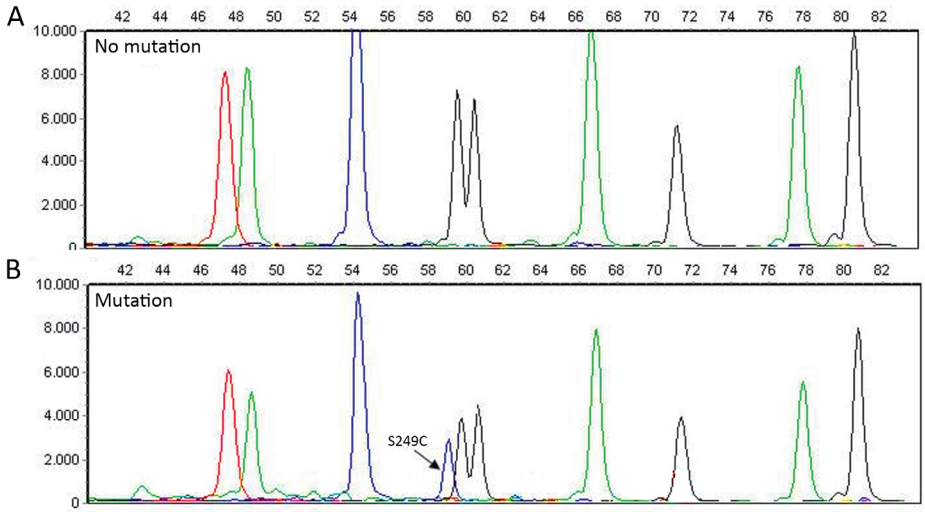

Figure 1.4 *FGFR3 SNaPshot analysis Wild-type (A) and Mutant (B)*

the RAS-MAPK pathway and appear to be mutually exclusive with mutations in the *FGFR3* gene (Figure 1.5).[71-73] Assays detecting RAS mutations in voided urine have been shown to be able to detect (recurrent) BC.

Phosphatidylinositol-3,4 -bisphosphate 3-kinase (PIK3CA) gene mutations

The phosphoinositide 3-kinase Phosphatidylinositol-3,4 -bisphosphate 3-kinase (PIK3CA) encodes for the catalytic p110-alpha subunit of Phosphatidylinositol 3-Kinase (PI3K) class IA, which is active in the PI3K-AKT pathway. The PI3K-AKT pathway is responsible for coordinating a diverse range of cell functions including cell proliferation, survival, proliferation, migration and morphology (Figure 1.5).[74, 75] Mutations in the *PIK3CA* gene are described in different tumor types such as colon, stomach, endometrium, ovary, thyroid, breast and glioblastomas.[76] Also a high mutation frequency is observed in bladder cancer ranging between 13% and 27% and these mutations often coincide with *FGFR3* mutations.[73, 77, 78] Kompier *et al* investigated the mutation frequency of *FGFR3*, *RAS* and *PIK3CA* in bladder cancer and showed that 88% of the non-muscle invasive bladder tumors harbored a mutation in at least one oncogene.[73] Mutations in *PIK3CA* are not prognostic but can be of use as a diagnostic tool.

Human Telomerase Reverse Transcriptase Gene Mutations

Telomerase is a ribonucleoprotein polymerase and the function of the enzyme is the preservation of the telomere ends by the addition of a telomere sequence repeat (TTAGGG). Normally, telomerase is only active in stem cells and repression of the enzyme in somatic cells will result in progressive shortening of the telomere ends. This will eventually lead to cellular senescence. There is cumulative evidence that cancer cells have the capability to overcome this senescence,

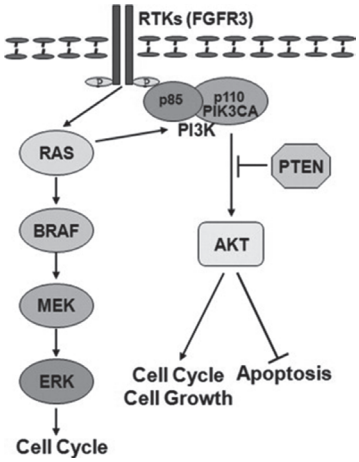

Figure 1.5 *RAS-MAPK and PI3K-AKT pathway. Source: Juanpere et al, Hum Pathol,* ***43:*** *1573, 2012*

leading to unlimited cell division[79, 80] Recently, somatic mutations in the gene coding for telomerase reverse transcriptase have been found in melanoma and in a small number of other tumors.[81, 82] The presence of *TERT* mutations in bladder cancer and their relation to patient outcome are studied in this thesis. Also, the ability of *TERT* mutation analysis for the detection of recurrences in urine is investigated.

Methylation analysis

DNA methylation is an epigenetic aberration that may affect gene expression without a change in the nucleotide sequence. DNA methylation plays a critical role in genomic imprinting, X-chromosome inactivation, and the regulation of tissue-specific gene expression.[83, 84] DNA methylation is the addition of a methyl group to the 5'-carbon of cytosine in a CpG sequence catalyzed by DNA methyltransferase. The CpG dinucleotides tend to cluster in so-called CpG islands. These CpG islands are defined as regions of more than 200 bases consisting of at least 50% G and C nucleotides and a ratio of observed to statistically expected CpG frequencies of more than 0.6.[85] Approximately 60% of the gene promoters contain CpG islands, which are usually unmethylated and allow gene transcription. Aberrant DNA promoter hypermethylation, causing transcriptional silencing of tumor suppressor genes, is found in different tumor types (Fig 1.6). Gonzalez-Zulueta *et al* first described hypermethylation in bladder cancer.[86] Since this initial study multiple genes with aberrant methylation have been identified in BC and showed association with age, gender, tumor location, stage, recurrence rate and progression.[87-89] Aberrant methylation has also been detected in voided urine of BC patients and appears to be more sensitive for tumor diagnosis than urine cytology.[90-94]

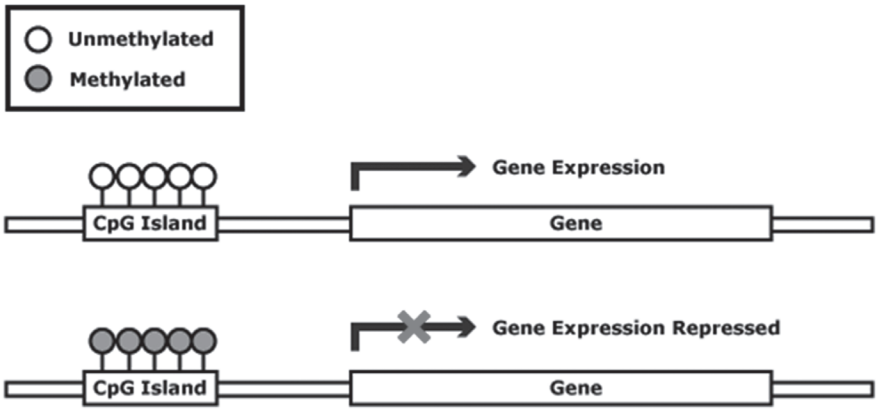

Figure 1.6 Aberrant promoter methylation leading to gene silencing. Source: www.uscf.edu

Microsatellite analysis

Microsatellites are short repetitive segments of DNA that are frequently observed throughout the genome. The repeated unit is 1 to 6 nucleotides long. The frequency of the repeated sequence can be highly variable and often differs between the maternal and paternal allele. Therefore, it could be used for the detection of loss of heterozygosity (LOH). LOH is the loss of part of the chromosome and is frequently observed in different types of cancer. Loss of the long arm of chromosome 9 (9q) is thought to be an early and important event in bladder carcinogenesis. Also loss of chromosome 8, 10, 11 and 17 are frequently found in BC.[95] Mao *et al* was the first describing the use of microsatellite analysis (MA) for the detection of primary BC in urine.[96] Shortly thereafter, Steiner *et al* investigated whether MA could also be used for the follow-up of BC patients.[97] Thereafter, many studies have been followed with sensitivity ranging from 75-92% and specificity between 79-100% for the detection of BC in patients under surveillance.[41] A disadvantage of the MA is the need of blood as reference and moreover the technique is laborious and expensive. Therefore, van Tilborg *et al* developed a MA for the detection of recurrent BC without the need for blood as reference.[98] This microsatellite analysis consisted of 12 probes for the detection of LOH in regions of chromosome 8, 9, 10, 11, and 17, resulting in a sensitivity of 58% for the detection of recurrent NMIBC in voided urine. Selection of six markers slightly reduced sensitivity to 55%. A smaller panel of markers without the need for blood analysis will reduce costs and workload.

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SCOPE OF THE THESIS

The general scope of this thesis is the assessment of the clinical value of molecular markers in the diagnosis, surveillance and prognosis of bladder cancer (BC). The introduction in **chapter 1** gives a background of BC, including clinical presentation, diagnosis, treatment, current follow-up and a summary of molecular markers discussed in this thesis.

Predicting prognosis in patients with NMIBC is highly dependable on clinical and pathological parameters, with stage and grade as important prognosticators. Yet, pathological assessment is subject to high interobserver and intraobserver variability. Molecular markers could contribute to a more accurate and objective review. In **chapter 2** we show the validation of four prognostic methylation markers, previously determined by Kandimalla *et al*. Based on the methylation data, new molecular grades are defined that predict clinical outcome. In **chapter 3** we investigate the presence of *TERT* mutations in bladder cancer and their relation to prognosis and patient outcome. Furthermore, we will evaluate the use of the *TERT* mutation analysis for recurrence detection in urine.

The third part evaluates the use of urinary markers for initial BC diagnosis and follow-up of patients with primary NMIBC. In **chapter 4** we design a bladder cancer-specific MS-MLPA (BC MS-MLPA) including genes that are frequently methylated in bladder cancer. This assay will be tested on follow-up urines from patients with primary NMIBC to assess the true additional value of the BC MS-MLPA during follow-up. In **chapter 5** we attempt to improve the detection rate of recurrent BC by combining different urinary assays. Pitfalls from chapter 5 will be used to improve recurrence detection in **chapter 6**, with a new combination of urinary assays. **Chapter 7** focuses on the role of molecular markers in diagnosing BC in patients presenting with hematuria. We investigate the discriminative ability of a set methylation markers in combination with clinical variables in patients presenting with hematuria. Finally, a predictive model will be developed that differentiates between low chance and high chance for BC.

In the final part we investigate BC in patients <20yr. The incidence of BC <20yr is extremely low and therefore very limited data is available, regarding this particular age-group. Conflicting results have been published, yet tumors from these patients seem to lack genomic aberrations that are commonly found in the elderly. In **chapter 8** BC from patients <20yr are investigated with methylation markers for polycomb group target genes (PcG's). PcG's play an important role in embryogenesis and previous research showed the methylation of PcG's is frequently observed in different cancer types. We presumed that this may be an early event in carcinogenesis and therefore it might be observed in tumors from young patients. In **chapter 9** tumors from the same patient group are examined for mutations that are commonly found in the elderly, such as the *FGFR3*, *RAS* and *PIK3CA* mutation.

PART II

MOLECULAR MARKERS AND BLADDER CANCER PROGNOSIS

Chapter 2

Stratification based on methylation of *TBX2* and *TBX3* into three molecular grades predicts progression in patients with pTa bladder cancer

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ABSTRACT

Objective | The potential risk of recurrence and progression in patients with non-muscle invasive bladder cancer necessitates follow-up by cystoscopy. The risk of progression to muscle invasive bladder cancer is estimated based on the European Organisation of Research and Treatment of Cancer score, a combination of several clinicopathological variables. However, pathological assessment is not objective and reproducibility is insufficient. The use of molecular markers could contribute to the estimation of tumor aggressiveness. We recently demonstrated that methylation of *GATA2, TBX2, TBX3*, and *ZIC4* genes could predict progression in Ta tumors. In this study we aimed to validate the markers in a large patient set using DNA from formalin-fixed and paraffin embedded tissue.

Material and methods | PALGA: the Dutch Pathology Registry was used for patient selection. We included 192 patients with pTaG1/2 bladder cancer of whom 77 experienced progression. Methylation analysis was performed and Log-rank analysis was used to calculate the predictive value of each methylation marker for developing progression over time.

Results | Log-rank analysis showed better progression-free survival in patients with low methylation rates compared to patients with high methylation rates for all markers ($p<0.001$) during a follow-up of ten-years. The combined predictive effect of the methylation markers was analyzed with the Cox-regression method. In this analysis, *TBX2*, *TBX3* and *ZIC4* were independent predictors of progression. Based on the methylation status of *TBX2* and *TBX3,* patients were divided into three new molecular grade groups. Survival analysis showed that only 8% of patients in the low molecular grade group progressed within five years. This was 29% and 63% for the intermediate and high molecular grade groups.

Conclusions | This new molecular grade based on the combination of *TBX2* and *TBX3* methylation is an excellent marker for predicting progression to muscle-invasive bladder cancer in patients with primary pTaG1/2 bladder cancer.

INTRODUCTION

Bladder cancer is the fifth most common malignancy in the western world and presents a significant health problem.[1] Clinically, 75-80% of bladder tumors are diagnosed as non-muscle invasive disease (Ta, T1, and Tis), while the remaining part is muscle invasive (T2-T4). Ta tumors represent the largest group of non-muscle invasive disease, accounting for approximately 70%.[2] These tumors are limited to the urothelial layer and are treated by transurethral resection. Overall, prognosis of these patients is favorable, especially for patients with G1/G2 disease. Although, high recurrence rates are observed, progression rates in patients with primary pTaG1/2 bladder cancer remain limited, ranging from 1-6%.[3, 4] Yet, due to the high mortality rates associated with muscle invasive bladder cancer, a life-long follow-up by cystoscopy is required. Cystoscopy is an expensive and invasive procedure, causing pain and discomfort to the patient.[5] The long survival of non-muscle invasive bladder cancer patients makes bladder cancer one of the most costliest cancers.[6] Costs and patient burden could be reduced by lowering the number of cystoscopies in patients with pTaG1/2 disease. Hence, correct risk stratification is necessary in order to safely adjust the surveillance protocol. Currently, the risk of progression is highly based on the pathological review. Yet, the reproducibility of pathology is modest and this may result in unwanted variations in patient management.[2, 7-9] Therefore many studies have been performed investigating new prognostic markers for the prediction of progression.[10-12] Nevertheless, there are still no accurate biomarkers available to predict progression in this large subgroup of patients.

Epigenetic aberrations, such as DNA hypermethylation, play an important role in the formation of many carcinomas and may serve as a cancer biomarker in the prediction of disease progression.[13-15] Recently, Kandimalla *et al* studied genome-wide hypermethylation of CpG islands in association with tumor progression.[16] They investigated 238 unique CpG islands in pTaG1/2 tumors with and without disease progression, i.e. progression to muscle invasive disease, metastasis or dead of disease. Four different CpG islands were selected, that were significantly associated with tumor progression, i.e. *GATA2, TBX2, TBX3*, *ZIC4*. In this study, the aim was to validate the clinical relevance of these four selected markers in a large set of patients with pTaG1/2 bladder cancer with and without progression.

MATERIAL AND METHODS

Patient and tissue collection

Patients with primary pTaG1/G2 bladder cancer were included. The nationwide network and registry of histopathology and cytopathology in the Netherlands (PALGA)[17] was searched for patients with progression of disease after resection of a primary pTaG1/G2 tumor and the tissue block and patient data were requested from the indicated pathology archives. Tumor tissue was used according to the code of secondary use of human tissue (www.federa.org). Progression was defined as progression to muscle-invasive disease, metastases or dead of disease. Patients

with pTaG1/G2 disease without progression were retrieved from our own pathology archive. All available cases were reevaluated by an expert uropathologist (GvL).

DNA Isolation and methylation analysis

Formalin-fixed paraffin-embedded tumor tissue was manually dissected from 20µm tissue sections based on an accompanying HE-stained section. Tumor tissues were dewaxed with xylene and ethanol. DNA was extracted using the QIAamp mini and Blood kit (Qiagen) according to manufacturer's protocol.

Methylation analysis of *GATA2, TBX2, TBX3 and ZIC4* was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, California, USA) according to the manufacturer's protocol. As described by Kandimalla *et al*, DNA was treated with sodium bisulfite, followed by a bisulfite-specific PCR for the four regions of interest. For each PCR reaction a DNA input of 10ng and a PCR primer concentration of 20 pM was required. After PCR, a SNaPshot analysis was performed, using probes that annealed to the PCR product adjacent to the cytosine of interest. Probes were extended with a labelled dideoxynucleotide and the products were analysed on an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a methylated cytosine. Primers and probes are given in table 2.1. For each gene, the methylation percentage was calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peaks multiplied by 100%.[16]

Table 2.1: Primer and probe sequences

BSP primers						
ZIC4	TTTTTATTTTTTGAGGGTAAATATTTAGTA					
	ATCTCCAAAAAACCTCTAAAACAAC					
TBX2	GTGGGGTTTTGGAATTTAGAATAGT					
	AACCACAAACTTAACCATCCACTAC					
TBX3	TTTGTTAATGGTTTGTAAATTT					
	AAAATAAATTTATCACCCACC					
GATA2	TATTGTTTTGTGTTTTTGGG					
	AACAAATTACAAACAAATTATACCTAA					
SNaPshot probes						
Probe	**Sequence (5'-> 3')**	**Size (bp)**	**Strand**	**UM**	**MT**	**µM***
ZIC4	T10 TAAATTTTGTTTAAATTTTT	30	sense	T	C	0,2
TBX2	T45 GTTGATGGATATTGTAGT	63	sense	T	C	0,2
TBX3	T13 AATTTTGGGATTAAAAG	30	sense	T	C	0,2
GATA2	T20 ACAAACAAATTATACCTAAC	40	antisense	A	G	0,2

** Concentration per PCR reaction,*
BSP= Bisulfite-specific PCR

Statistical analysis

Statistical analyses were completed using the IBM SPSS statistics 20. Chi-square test was used to determine relationships between different variables. Survival was estimated according to the Kaplan-Meier method and compared using log-rank tests. Multivariate Cox-regression analysis with backward elimination was used to calculate the independent prognostic value of the methylation markers. P-values <0.05 were considered statistically significant.

RESULTS

Patients and tumor characteristics

A total of 192 patients with primary pTa G1/G2 bladder cancer were included in this study. There were 115 patients without progression with a median follow-up of 84 months. 77 patients had progression of disease with a median time to progression of 38 months. Of these, 66/77 (86%) patients developed progression within 10 years. Patient and tumor characteristics are depicted in table 2.2. Male: female ratio in both groups was 3:1. Overall, primary tumors of the progressors were higher grade (G2) compared to the primary tumors of the non-progressors, 70% vs. 42% respectively (p<0.001). Seven patients were originally graded as low-grade according the WHO2004 grading system. These seven patients were excluded in multivariate Cox-regression analysis.

Table 2.2: Patient and tumor characteristics

		Progressors n=77	Non-progressors n=115	
Age				
	Mean (range)	68 (47-85)	63 (23-86)	
		n (%)	**n (%)**	*p-value*
Sex				
	Male	59 (77)	84 (73)	0.58
	Female	18 (23)	31 (27)	
Stage				
	Ta	77 (100)	115 (100)	-
Grade				
	Grade 1	18 (23)	64 (56)	<0.001
	Grade 2	54 (70)	49 (42)	
	Low Grade	5 (7)	2 (2)	

Validation of the prognostic markers for prediction of progression

Tumor DNA was analyzed for methylation of *GATA2*, *TBX2*, *TBX3* and *ZIC4*. Methylation analysis failed in 41/768 (5%), i.e. *GATA2* n=0, *TBX2* n=20, *TBX3* n=14 and *ZIC4* n=7. The efficacy of each gene was determined by calculating the area under the receiver-operating curve. The area under the curve for *GATA2* was 0.803, *TBX2* 0.644, *TBX3* 0.785 and *ZIC4* 0.692. A methylation percentage cut-off was selected with the best combination of sensitivity and specificity for each individual marker (Figure 2.1). Cut-off values, sensitivity and specificity are given in table 2.3.

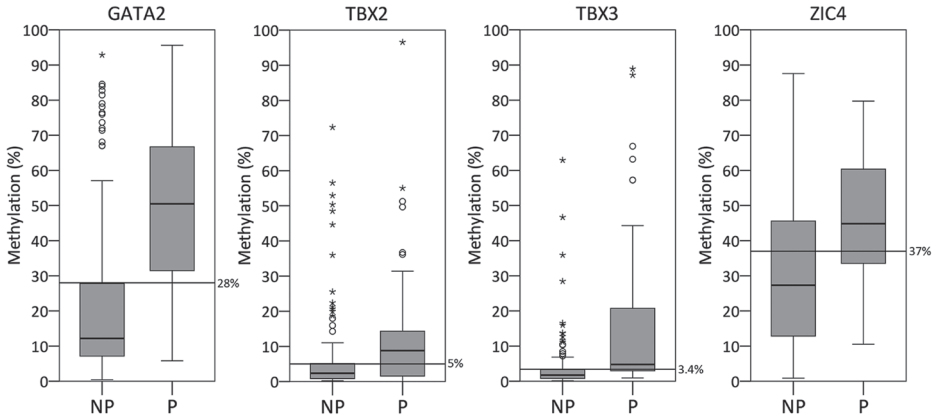

Figure 2.1 Boxplots indicating the range of gene methylation % regarding the progression status: progressors (P) and non-progressors (NP). The horizontal line indicates the cut-off.

Table 2.3: Methylation marker performance for predicting disease progression

Methylation marker	Cut-off	Sensitivity	n	Specificity	n	AUC
TBX3	3.4%	71%	51/72	76%	80/106	0.785
TBX2	5.0%	63%	46/73	66%	66/99	0.644
ZIC4	37%	70%	53/76	64%	70/109	0.692
GATA2	28%	78%	60/77	75%	86/115	0.803

To determine the predictive value of the methylation markers for developing progression over time, Log-rank analysis was performed based on a follow-up period of ten years. 66/77 (86%) of the progressors developed disease progression within ten years of follow-up. Figure 2.2 shows the resulting survival graphs for grade and the four methylation markers. Patients with a G1 tumor or low methylation of *TBX2*, *TBX3*, *GATA2* and *ZIC4* had a significantly better progression-free survival, p<0.001 (Figure 2.2). In order to calculate the combined effect of the methylation markers for predicting progression over time, multivariate Cox-regression analysis with backward elimination was performed. This analysis based on *TBX2*, *TBX3*, *GATA2* and *ZIC4* in combination with grade showed the clear independent prognostic ability of *TBX3* for the risk of progression within ten years of follow-up time (Table 2.4, multivariate analysis step #1). After removing *GATA2* as predictor from

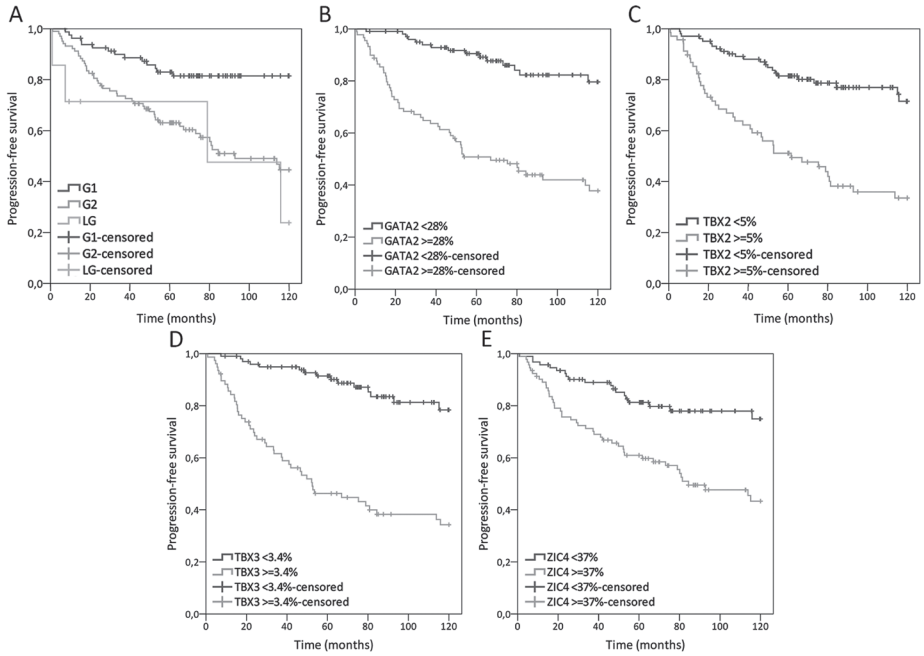

Figure 2.2 Kaplan-Meijer curves showing the time to progression of patients with primary TaG1/G2 BC, according to the grade (A) given at time of diagnosis and according to the methylation status of GATA2 (B), TBX2 (C), TBX3 (D) and ZIC4 (E) in the primary tumor. A: G1 vs. G2 p<0.001, G1 vs. LG p<0.001 and G2 vs. LG p=0.355. B-E: patients with low methylation status showed better progression-free survival compared to patients with high methylation status (p<0.001).

the model *TBX3* and *TBX2* appeared the best predictors (multivariate analysis step #2). Based on the methylation status of *TBX2* and *TBX3,* a new molecular grading system was defined. Patients were assigned to the high molecular grade group in case both markers were positive. The intermediate grade group group included patients with one marker positive. The low molecular grade group consisted of patients with none of the markers positive. Disease progression occurred in 32/43 (74%) of the patients in the high molecular grade group and in 20/49 (40%) patients of the intermediate molecular grade group. In comparison, only 9/73 (12%) patients in the low molecular grade group showed disease progression. The majority of patients developed progression within 5 years of follow-up: 27/43 (63%) of the patients from the high molecular grade group compared to 14/49 (29%) of the intermediate grade group and 6/73 (8%) of the low molecular grade group. Log-rank analysis showed significantly better progression-free survival in patients assigned to the low molecular grade group compared to patients at the intermediate molecular grade group and the high molecular grade group. Furthermore, patients in the intermediate grade group showed better progression-free survival than patients in the high molecular grade group (low molecular grade vs. intermediate molecular grade p = 0.04, low molecular grade group vs. high molecular grade p <0.001, intermediate molecular grade vs. high molecular grade p < 0.001) (Figure 2.3).

Table 2.4: Univariate and multivariate progression-free survival analysis

		Univariate analysis			Multivariate step #1			Multivariate step #2		
Variable		No of pts	events	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Grade	G1	82	14	<0.001	1.824	0.957 - 3.474	0.068	1.882	0.988 - 3.586	0.054
	G2	103	48							
TBX2	< 5.0% methylated	103	23	<0.001	1.656	0.922 - 2.975	0.091	1.873	1.069 - 3.283	0.028
	≥ 5.0% methylated	69	41							
TBX3	< 3.4% methylated	101	15	<0.001	3.522	1.788 - 6.936	<0.001	4.087	2.149 - 7.772	<0.001
	≥ 3.4% methylated	77	47							
ZIC4	< 37% methylated	93	19	<0.001	1.786	0.985 - 3.237	0.056	1.873	1.038 - 3.379	0.037
	≥ 37% methylated	92	46							
GATA2	< 28% methylated	103	15	<0.001	1.605	0.802 - 3.213	0.181	-	-	-
	≥ 28% methylated	89	51							

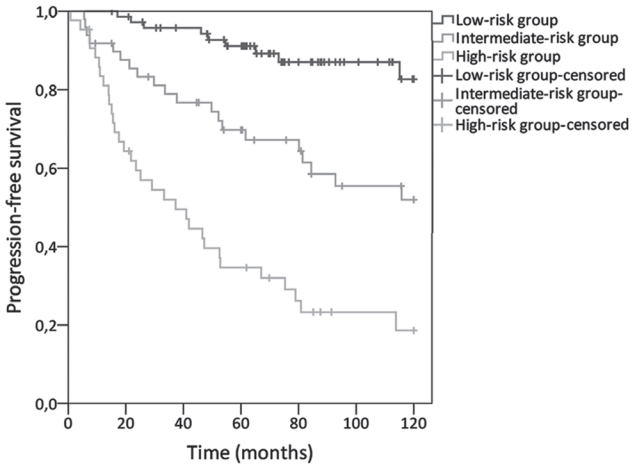

Figure 2.3 *Patients stratified according to methylation status of TBX2 and TBX3 into three molecular grade groups: Low molecular grade (lmG) both markers negative, intermediate-molecular (imG) one marker positive and high molecular grade (hmG) both markers positive. A follow-up time of maximum ten years was selected.*
Supplementary figure 2.1 *Kaplan-Meijer curves showing the time to progression of patients with primary TaG1/G2 bladder cancer, according to the pathological reassessment (A) Revised grade by WHO1973 (B) Revised grade by WHO2004.*

Reevaluation of the pathological review

All cases were reviewed by an expert uropathologist. Seventeen were not available. This reevaluation was not completely unbiased because the pathologist was aware that the series comprised many tumors that had progressed. Reassessment of the histological slides revealed a dissimilarity of 12% (21/175) in staging and of 54% (95/175) in revised grading by the WHO1973 system. 80/175 (45%) tumors were upgraded and 8% (15/175) of the tumors were downgraded. Furthermore, tumors of the progressors were higher graded and staged compared to the group of non-progressors, p<0.001 (table 2.5). Revised grade by the WHO2004-system showed more high-grade tumors in the progressor-group (p<0.001).

Table 2.5: Tumor characteristics after reassessment of histological slides

	Progressors	Non-progressors	
	n=77	n=98	
Reassessment	**n (%)**	**n (%)**	*p-value*
Stage			
Papilloma	2 (3)	1 (1)	
Ta	56 (73)	95 (97)	<0.001
T1	17 (22)	2 (2)	
T2	1 (1)	-	
Tis	1 (1)	-	
Revised grade by WHO 1973*			
Papilloma	2 (3)	1 (1)	<0.001
G1	3 (4)	21 (22)	
G2	45 (59)	66 (67)	
G3	26 (34)	10 (10)	
Revised grade by WHO 2004*			
Papilloma	2 (3)	1 (1)	<0.001
PUNLMP	2 (3)	8 (8)	
Low Grade	20 (26)	57 (58)	
High Grade	52 (68)	32 (33)	

** Without Tis n=1*

Log-rank analysis based on the revised WHO1973 grade revealed better progression-free survival in G1 vs. G2 and G2 vs. G3 (Supplementary Figure 2.1 A: G1 vs G2 p=0.026, G1 vs G3 p<0.001 and G2 vs. G3 p<0.001). WHO2004 grading also showed a worse progression-free survival for patients with a high-grade tumor compared to patients with a low grade or PUNLMP tumor (Supplementary Fig 2.1B: HG vs. LG <0.001, LG vs. PUNLMP p=0.534, and HG vs PUNLMP p=0.035). Next, we performed multivariate Cox regression analyses with backward elimination to compare revised grade with the molecular markers. These results are depicted in the supplementary tables 2.1 and 2.2. Compared to both grading systems *TBX3* and *TBX2* remained the best predictors for progression.

DISCUSSION

Although most important predictors for disease progression in patients with non-muscle invasive bladder cancer are stage, grade and carcinoma in situ[18], patients with primary low stage and low grade bladder cancer still have a 1-6% chance of disease progression according the European Association of Urology guidelines.[3] In order to safely reduce the number of cystoscopies in patients with low grade non-muscle invasive bladder cancer, risk prediction is necessary. Up to now, no sensitive markers are available to predict progression in this specific patient group. Recently, Kandimalla *et al.* proved the prognostic ability of the methylation markers *TBX2*, *TBX3*, *GATA2* and *ZIC4* using fresh frozen tumor tissue.[16] In this more extensive validation these markers again proved to be highly sensitive for prediction of progression in low-grade pTa disease. Patients with low methylation percentages of *TBX2*, *TBX3*, *GATA2* and *ZIC*4 showed better progression-free survival in univariate Log-rank analysis. Multivariate Cox-regression analysis showed an independent increased risk for progression in patients with high methylation ratios of *TBX2*, *TBX3* and *ZIC4*. New molecular grading based on the methylation status of *TBX2* and *TBX3* into three risk groups resulted in accurate prediction of progression risk, with better progression-free survival in patients in the low risk group and intermediate risk group. In addition, this validation was carried out on DNA isolated from formalin-fixed paraffin embedded tissue.

The four investigated genes*, TBX2*, *TBX3*, *GATA2* and *ZIC4,* encode transcription factors that are important in the regulation of developmental processes and gene expression is more frequent in non-muscle invasive bladder cancer compared to muscle invasive disease.[19, 20] This could be linked to our results in which aggressive tumors that progressed to muscle-invasive bladder cancer showed more methylation than the non-progressive tumors. Furthermore, the most significant markers, *TBX2* and *TBX3*, downregulate the p53-pathway by inhibiting the expression of the *ARF* and *CIP1* gene.[21] Mutation of the *p53* gene is a common phenomenon in muscle invasive bladder cancer. Methylation of *TBX2* or *TBX3* could be a different way to inhibit the *p53*-pathway. Therefore, we propose that methylation of *TBX2* and *TBX3* characterizes the more aggressive non-muscle invasive bladder cancer, which are more similar to the muscle-invasive bladder cancer.

Multiple studies previously reported the prognostic ability of methylation markers based on DNA isolated from formalin-fixed paraffin-embedded tissue.[22] Friedrich *et al* found that methylation of *TIMP3* was associated with a better progression-free survival in patients with pT1 and pTa tumors.[23] Yan *et al* showed that methylation of *RUNX3* in combination with G3 increased the chance of disease progression.[24] According to Yates *et al.* methylation of *E-cadherin*, *TNFRSF25*, *EDNRB*, *RASSF1A* and *APC* were significantly associated with progressive disease.[15] Yet, these studies all used a mixture of pT1 and pTa tumors. Our study specifically focused on patients with low-grade pTa disease. These patients have the lowest chance of developing progression and therefore, adjustment of the surveillance protocol will be most beneficial in this particular patient group.

The progression scores as recommended in the European Association of Urology guidelines are currently calculated according to the European Organisation of Research and Treatment of Cancer risk scores developed by Sylvester *et al.*[3] The pathological review plays an important role in this prediction model. However, this pathological assessment highly depends on the experience of the pathologist and suffers from high interobserver and intraobserver variation.[8, 25] Upgrading and upstaging could increase the 5-year progression risk up to 45% according to the European Organisation of Research and Treatment of Cancer risk calculator. This emphasizes the importance of accurate pathological assessment. This study again proved the high interobserver variation with significant more high grade tumors among the progressors after pathological reassessment. Yet, it should be emphasized that the pathological review could be biased. Although, the slides were blinded, the pathologist was aware of the fact that there was a high percentage of aggressive tumors in this study. Therefore, we decided to use the original pathological grade as inclusion criteria, since this is the diagnosis on which further treatment was based. Patient outcome is a result of this original review and the treatment choices made.

The use of molecular markers could facilitate the pathologist's estimation of tumor aggressiveness. A previous study by van Rhijn *et al* investigated the additional value of molecular markers to the pathological assessment. Molecular grading based on the combination of *FGFR3* mutation status and *MIB1* expression resulted in better risk prediction compared to conventional pathological grade.[26] The addition of the methylation markers to *FGFR3* and *MIB1* might further increase the predictive accuracy. The methylation analysis is an easy test and could be performed in a standard laboratory. Furthermore, the SnapShot method proved to be highly reproducible.[27]

A limitation of this study is the retrospective design. Due to the low incidence of progression in low grade Ta disease a prospective study design is difficult to establish. Therefore, the distribution of patients is not representative for the clinical practice, since the actual percentage of patients with low-grade non-muscle invasive bladder cancer and disease progression will be much lower in the clinical setting. However, the predictive risk ability of the methylation markers still holds after a second validation and therefore, we believe that these results could be translated to the clinic.

CONCLUSIONS

This study represents an independent validation of pTa-prognostic markers in a large set of patients. These markers proved again to be accurate risk predictors for progression in patients with primary low-grade pTa bladder cancer. New molecular grading based on the methylation status of *TBX2* and *TBX3* resulted in sensitive risk prediction for progression. Determining DNA methylation is a highly reproducible method as we showed previously.[27] Hence these markers present a valuable tool for assessing risk of progression in patients presenting with TaG1/G2 tumors.

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SUPPLEMENTARY FILES

Supplementary table 2.1: multivariate progression-free survival analysis based on the methytlation markers and re-grade WHO1973

	Multivariate analysis step #1			Multivariate analysis step #2			Multivariate analysis step #3		
Variable	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Revised Grade WHO 1973	1.335	0.857-2.081	0.202	1.357	0.873-2.112	0.175	-	-	-
TBX2	1.616	0.923-2.829	0.093	1.748	1.013-3.019	0.045	1.823	1.061-3.130	0.030
TBX3	3.662	1.909-7.028	<0.001	4.247	2.294-7.861	<0.001	4.638	2.543-8.460	<0.001
ZIC4	1.683	0.942-3.010	0.079	1.651	0.992-2.957	0.092	1.812	1.029-3.193	0.040
GATA2	1.513	0.792-2.894	0.210	-	-	-	-	-	-

Supplementary table 2.2: multivariate progression-free survival analysis based on the methytlation markers and re-grade WHO2004

	Multivariate analysis step #1			Multivariate analysis step #1			Multivariate analysis step #3		
Variable	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Revised Grade WHO 2004	1.891	1.155-3.096	0.011	1.961	1.205-3.191	0.007	2.126	1.322-3.419	0.002
TBX2	1.789	1.023-3.129	0.041	1.889	1.095-3.258	0.022	1.936	1.121-3.344	0.018
TBX3	3.840	2.013-7.324	<0.001	4.232	2.301-7.780	<0.001	4.439	2.420-8.148	<0,001
ZIC4	1.483	0.824-2.670	0.189	1.458	0.811-2.622	0.208	-	-	-
GATA2	1.327	0.690-2.552	0.396	-	-	-	-	-	-

Chapter 3

Telomerase reverse transcriptase promoter mutations in bladder cancer: high frequency across stages, detection in urine and lack of association with outcome

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ABSTRACT

Objective | To investigate *TERT* mutation frequency, spectrum, association with expression and clinical outcome, and potential for detection of recurrences in urine in patients with urothelial bladder cancer.

Material and methods | A set of 111 UBCs of different stages was used to assess *TERT* promoter mutations by Sanger sequencing and TERT messenger RNA (mRNA) expression by reverse transcription-quantitative polymerase chain reaction. The two most frequent mutations were investigated, using a SNaPshot assay, in an independent set of 184 non–muscle-invasive and 173 muscle-invasive UBC (median follow-up: 53 mo and 21 mo, respectively). Voided urine from patients with suspicion of incident UBC (n = 174), or under surveillance after diagnosis of non–muscle-invasive UBC (n = 194), was tested using a SNaPshot assay. The outcome measurements were: Association of mutation status with age, sex, tobacco, stage, grade, fibroblast growth factor receptor 3 (*FGFR3*) mutation, progression-free survival, disease-specific survival, and overall survival.

Results | In the two series, 78 of 111 (70%) and 283 of 357 (79%) tumors harbored *TERT* mutations, C228T being the most frequent substitution (83% for both series). *TERT* mutations were not associated with clinical or pathologic parameters, but were more frequent among *FGFR3* mutant tumors (p = 0.0002). There was no association between *TERT* mutations and mRNA expression (p = 0.3). Mutations were not associated with clinical outcome. In urine, *TERT* mutations had 90% specificity in subjects with hematuria but no bladder tumor, and 73% in recurrence-free UBC patients. The sensitivity was 62% in incident and 42% in recurrent UBC. A limitation of the study is its retrospective nature.

Conclusions | Somatic *TERT* promoter mutations are an early, highly prevalent genetic event in UBC and are not associated with TERT mRNA levels or disease outcomes. A SNaPshot assay in urine may help to detect UBC recurrences.

INTRODUCTION

Urothelial bladder cancer (UBC) is heterogeneous at the clinical, pathologic, and genetic levels. Approximately 75% of newly diagnosed tumors are non–muscle invasive (Ta, Tis, and T1); most of them recur and 15–20% progress to invade muscle. The remaining 25% of patients present with muscle-invasive tumors and have a 5-yr survival of <50%.[1-3] Fibroblast growth factor receptor 3 (*FGFR3*) is the most commonly mutated gene in UBC, with an overall frequency of 60%.[4-6] Mutations in Harvey rat sarcoma viral oncogene homolog (*HRAS*), neuroblastoma RAS viral (v-ras) oncogene homolog (*NRAS*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) are less frequent.[6, 7] *FGFR3* mutations are associated with stage and grade and are very common in pTa and grade 1/2 tumors. This makes *FGFR3* mutation analysis in voided urine a useful tool for diagnosing recurrent disease after a primary non–muscle-invasive bladder cancer (NMIBC).[8, 9]

Telomerase increases telomere length at chromosome ends. This activity is crucial for proliferating cells: Without telomerase, chromosomes are shortened every cell-division cycle, leading to replicative senescence and genomic instability.[10, 11] Telomerase is active in stem cells but becomes downregulated in differentiated cells in somatic tissues. By contrast, it becomes reactivated in many tumors. Somatic hotspot mutations in the promoter of the gene coding for telomerase reverse transcriptase (TERT) catalytic subunit have recently been described in 71% of sporadic melanomas; germline mutations have also been reported in a melanoma kindred.[12, 13] The somatic mutations were mutually exclusive C-to-T transitions at nucleotides 1,295,228 (C228T) and 1,295,250 (C250T). These are the first hotspot somatic mutations reported in a gene promoter in human cancers and have been proposed to enhance TERT expression through the creation of novel binding sites for E-twenty-six (ETS)/ELK transcription factors.[12, 13] Nevertheless, direct proof for this mechanism is absent. Interestingly, Huang et al. reported the C228T mutation in three of three UBC cell lines[13] and, in a survey of different tumor types, *TERT* promoter mutations were also found in UBC, hepatocarcinoma, glioblastoma, oligodendroglioma, and myxoid liposarcomas, but not in breast, colorectal, and prostate cancers.[14] However, the number of bladder tumors analyzed was small and clinical or pathologic information was not available.[14] In this chapter, we investigate the frequency of *TERT* promoter mutations in two large series of UBC. In the first one, lacking outcome information, we assessed the relationship between *TERT* mutations and TERT messenger RNA (mRNA) expression; in the second, with patient follow-up, we assessed the relationship between mutations and outcome. Finally, we explored whether mutation detection in urine may be used to identify tumor recurrence.

MATERIAL AND METHODS

Patient characteristics

A set of 111 patients with associated frozen tumor samples came from the Spanish Bladder Cancer/EPICURO Study[15, 16] and from the Integrated Study of Bladder Cancer (ISBLAC). Clinical and sociodemographic information was retrieved from hospital records through a structured questionnaire. Subjects were defined as former smokers if they quit smoking ≥1 yr before the date of interview. A second set of 357 patients, with associated formalin-fixed paraffin embed-

Table 3.1: Characteristics of patients whose tumor samples were studied

		Spain (n=111)	The Netherlands, NMIBC (n=184)	The Netherlands, MIBC (n=173)
Characteristic	*Variable*	no. (%)	no. (%)	no. (%)
Age, yr, median		76	70	71
Sex	*Female*	13 (12)	46 (25)	28 (16)
	Male	98 (88)	138 (75)	145 (84)
Stage	*pTa + pTis*	63 (57)	107 (58)	–
	pT1	28 (25)	77 (42)	–
	pT2	13 (12)	–	72 (42)
	pT3	3 (3)	–	75 (43)
	pT4	3 (3)	–	26 (15)
Grade	*G1*	27 (24)	14 (8)	1 (1)
	G2	28 (25)	101 (55)	13 (7.5)
	G3	56 (51)	69 (37)	159 (92)
Follow-up, mo, median		–	53	21
End point	*Progression to MIBC*	–	90 (49)	–
	Local or distant progression to MIBC	–	–	78 (45)
	Died of disease	–	–	65 (37)
	Died of other causes	–	–	36 (21)
	Alive at follow-up	–	–	37 (21)
	Lost to follow-up	–	–	35 (20)
TERT status	*MT*	78 (70)	147 (80)	136 (79)
	WT	33 (30)	37 (20)	37 (21)
TERT mutations *	*C228T*	65 (83)	121 (82)	113 (83)
	C228A	1 (1.5)	3 (2)	3 (2)
	CC242/243TT	2 (2.5)	NA	NA
	C250T	10 (13)	23 (16)	20 (15)

MT = mutant; NMIBC = non–muscle-invasive bladder cancer; TERT = telomerase reverse transcriptase; WT = wild type.
** The C242T mutation was not assessable with the SNaPshot assay.*

ded (FFPE) tumor samples and follow-up information, came from the Erasmus Medical Center and other hospitals in the Netherlands. This cohort comprises 184 patients with primary NMIBC selected to contain a 50:50 distribution of cases with or without progression; the latter was defined by the development of MIBC. It also comprises 173 patients who have undergone a cystectomy for primary MIBC, stage pT2-4. Cause of death for patients in the MIBC cohort was determined from hospital records and/or direct contact with general practitioners. Staging and grading were performed according to the TNM 2002 classification and the three-grade 1973 World Health Organization classification. To confirm staging/grading and ensure uniformity of classification criteria, expert pathologists reviewed diagnostic slides from all tumor blocks. Table 3.1 shows the characteristics of patients whose tumor samples were studied.

For the urine study, samples were obtained from a consecutive, prospectively sampled collection of urine specimens from two centers (Hospital del Mar, Barcelona, Spain; and Erasmus MC, Rotterdam, Netherlands) participating in the European Community Seventh Framework

Table 3.2: Characteristics of tumors and patients used for urine diagnostic assays

Characteristic	*Variable*	The Netherlands	Spain
		no. (%)	no. (%)
Patients with primary tumor diagnosis	*Patients, no.*	45	90
Stage	*pTa*	39 (87)	53 (59)
	pTis		3 (3)
	pT1	5 (11)	21 (23)
	pT2-4	1 (2)	13 (15)
Grade	*G1*	17 (38)	27 (30)
	G2	26 (58)	17 (19)
	G3	2 (4)	46 (51)
Patients under surveillance after NMIBC diagnosis*	*Patients, no.*	118	76
With relapsing tumors			
Stage	*pTa*	78 (90)	40 (71)
	pTis	-	2 (4)
	pT1	2 (2)	7 (12)
	pT2-4	5 (6)	4 (6)
	pTx	2 (2)	3 (7)
Grade	*G1*	25 (29)	23 (35)
	G2	52 (60)	13 (23)
	G3	8 (9)	19 (32)
	Gx	2 (2)	1 (10)
Recurrence-free	*Patients, no./urine, no. samples, no.*	65/232	20/20

NMIBC = non–muscle-invasive bladder cancer.

** Relapsing tumors include both recurrences and progression detected in patients with initial diagnosis of NMIBC.*

program–funded "Prediction of bladder cancer disease course using risk scores that combine molecular and clinical risk factors" (UROMOL) study (EU-7FP UROMOL #201663). Samples were obtained prior to cystoscopy at the time when a primary UBC was diagnosed (*n* = 135), or from patients lacking a prior history of UBC who presented with hematuria for diagnostic work-up and lacked visible bladder tumors at cystoscopy (*n* = 39). Additional samples (*n* = 395) were obtained during surveillance for relapsed disease (recurrence or progression) in 194 patients with NMIBC. Primary tumor or recurrence was defined by the presence of a histologically proven tumor. Table 3.2 shows the characteristics of patients included in the urine study. All patients provided written informed consent. The ethics committees of the participating institutions approved all studies. Samples from the Netherlands were obtained according to the Federation of Dutch Medical Scientific Societies' Code for Proper Secondary Use of Human Tissues in the Netherlands (http://www.federa.org/).

Cell lines

UBC cell lines (Supplementary Table 3.1) were cultured under standard conditions and used for DNA extraction when at 70–80% confluence. All cultures were *Mycoplasma* free; their identity was tested by analysis of known genetic mutations.

DNA isolation and mutation analyses

Tumor cell-containing regions were macrodissected from frozen blocks or FFPE sections after examination of hematoxylin and eosin–stained sections. For MIBC cases, DNA was isolated from tumor obtained at the time of cystectomy or, in case of pT0, of incident transurethral resection. Tumor DNA and leukocyte DNA were extracted using the Qiagen kit for blood and tissue, according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Sanger sequencing of polymerase chain reaction (PCR) products was used to identify all possible mutations in the first set of patients (Fig. 1A). A 163-base pair (bp) PCR product encompassing the proximal *TERT* promoter was amplified (primers: CAGCGCTGCCTGAAACTC [forward] and GTCCTGCCCCTTCACCTT [reverse]); for some samples, a broader 488-bp region of the promoter was amplified (primers: AGCACCTCGCGGTAGTGG [forward], GGCCGATTCGACCTCTCT [reverse]). PCR conditions can be provided on request to the authors. *FGFR3* mutation assays were performed as described.[8] For the *TERT* mutational analysis in the second patient group, we designed a SNaPshot assay (Invitrogen Corp, Carlsbad, CA, USA) through a PCR (155 bp) covering the two most frequent mutations identified (primers: AGCGCTGCCTGAAACTCG [forward], CCCTTCACCTTCCAGCTC [reverse]), similar to the previously designed *FGFR3* mutation assay.[8] The PCR was followed by a single-nucleotide extension using probes annealing to the amplicon adjacent to the mutation site. Probe sequences were: T_{23}GGCTGGGAGGGCCCGGA for mutation 1,295,228 (C228T/A) and T_{39}CTGGGCCGGGGACCCGG for 1,295,250 (C250T) at 1 μM and 1.5 μM, respectively (ABI PRISM SNaPshot Multiplex Kit; Applied Biosystems, Foster City, CA, USA). The products were analyzed (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) with the label indicating the presence or absence of a mutation. Genescan

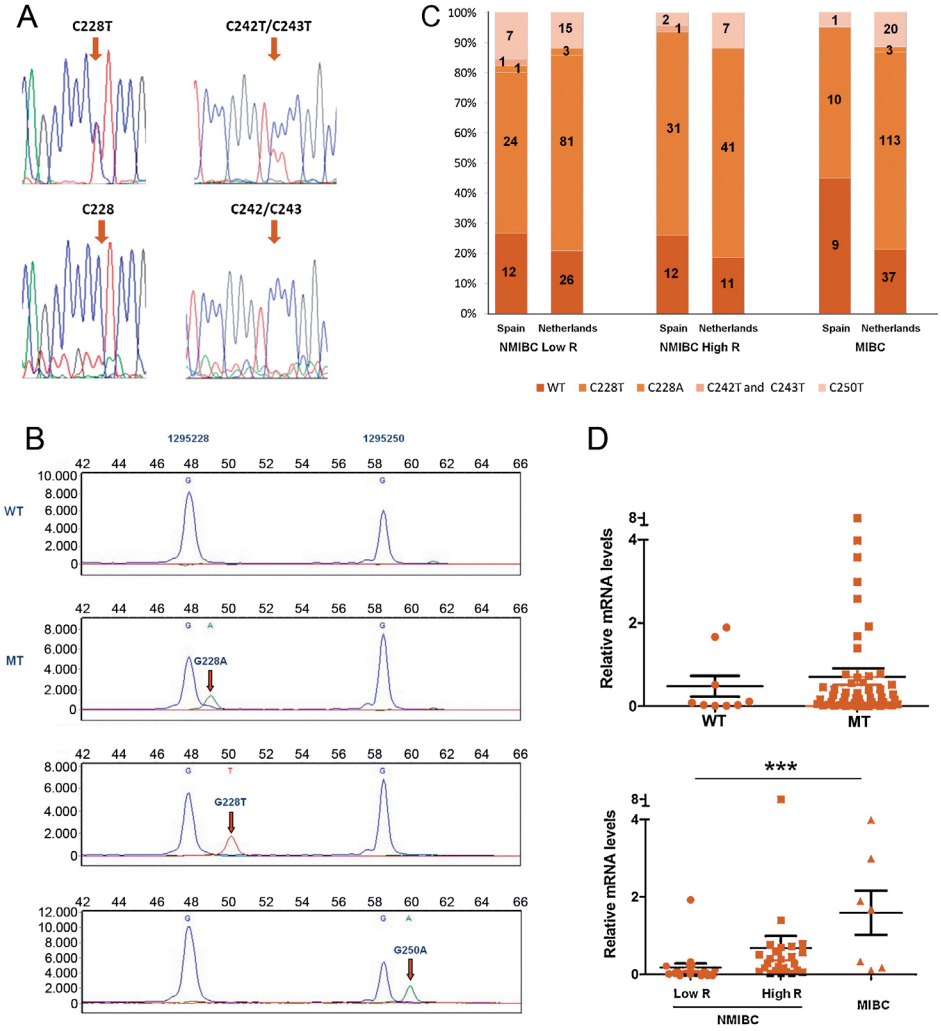

Figure 3.1 *Somatic telomerase reverse transcriptase (TERT) promoter mutations in urothelial bladder cancer (UBC). (A) The C228T and C242T/C243T mutations are detected in tumor DNA but not in leukocyte DNA (Sanger sequencing). (B) Mutation assay for the indicated nucleotides in the promoter of the TERT gene. Upper panel: Control sample with wild-type peaks for positions C228T and C250T (from left to right). The lower panels show mutant samples and the nature of the mutation has been indicated. Note that the assay detects the mutations from the complementary strand, hence the mutations are indicated as G228A, G228T, and G250A. (C) Distribution of TERT promoter mutations in UBC according to T and G. Tumors are categorized in three groups: low-risk non–muscle-invasive bladder cancer (NMIBC) (TaG1/G2) (Low R), high-risk NMIBC (TaG3, T1G2/G3) (High R), and MIBC (>T2) for Spain (Sanger sequencing) and the Netherlands (SNaPshot; Applied Biosystems, Foster City, CA, USA). Absolute numbers of cases are given for each mutation and group. (D) TERT messenger RNA levels in bladder tumors assessed by reverse transcriptase-quantitative polymerase chain reaction (n = 60); results show the comparison of wild type versus mutant tumors and the comparison of the three tumor groups according to T and G. *** $p \leq 0.001$. WT = wild type; MT = mutant type.*

Analysis Software v.3.7 (Applied Biosystems, Foster City, CA, USA) was used for data analysis (Fig. 1B). For urine DNA extraction and mutation analysis, cell pellets were washed twice with phosphate-buffered saline, after thawing, by centrifugation for 5 min at 6000 rpm (3000 g). DNA was extracted using the QiAamp Mini and Blood mini kit (Qiagen NV, Venlo, Netherlands) according to the manufacturer's protocol. *TERT* mutational analysis was performed using the SNaPshot assay. Reverse transcriptase-quantitative PCR (RT-qPCR) was used to assess TERT RNA expression.

Statistical and bioinformatics analyses

The Predictive Analytics Software v.19 (IBM Corp., Armonk, NY, USA) was used. Statistical differences were considered significant if $p < 0.05$. The chi-square and Fisher exact tests were used to determine the relationships between different variables. Statistical differences between groups were assessed using a nonparametric Wilcoxon test, as appropriate. The log-rank test was used for survival analysis. In the NMIBC group, all outcomes other than progression appear as censored in the Kaplan-Meier curve. Bioinformatics analysis of the *TERT* promoter sequence is detailed in the Supplement.

RESULTS

TERT promoter mutations in bladder cancer

We first analyzed the occurrence of mutations in UBC cell lines and tumors using Sanger sequencing of PCR products. Of 32 lines analyzed, 4 were wild type and 28 (87%) harbored the C228T ($n = 25$) or C250T ($n = 3$) mutations (Supplementary Table 3.1). The somatic nature of these mutations could not be evaluated due to lack of germline DNA.

Among the 111 UBCs from Spain, covering the full spectrum of the localized disease, 78 were mutated (70%) (Table 3.1). The most common mutation was C228T ($n = 65$), followed by C250T ($n = 10$); two additional rare mutations were C242T/C243T ($n = 2$) and C228A ($n = 1$). All mutations were mutually exclusive; their somatic nature was confirmed in 10 cases (Fig. 1A). The C250T mutation was significantly less common in UBC than in melanoma ($p = 0.00003$); the C228T/C229T mutation reported in melanoma was not found in UBC. *TERT* mutations were not associated with age, sex, or smoking. The frequency of *TERT* mutations was similar in low-risk NMIBC (TaG1 and TaG2; 33 of 45 [73%]), high-risk NMIBC (TaG3, Tis, T1G2, T1G3; 34 of 46 [74%]), and in MIBC (10 of 19 [53%]) ($p = 0.192$) (Fig. 1C) and in newly diagnosed versus recurrent tumors ($p = 0.716$). *TERT* mutations were significantly more frequent among *FGFR3* mutant tumors (43 of 49 vs 29 of 54; $p = 0.0002$).

We analyzed the two most frequent *TERT* mutations in an independent set of tumors from the Netherlands using a specifically designed SNaPshot assay; mutations were found to be mutually exclusive. A high mutation rate was confirmed in both NMIBC (147 of 184 [80%]) and MIBC (136 of 173 [79%]) (Fig. 1C). To assess heterogeneity, we analyzed *TERT* mutations using

the SNaPshot assay in multiple (median: 4; range: 2–5) regions of nine NMIBCs. We observed general consistency among the regions, suggesting a low level of intraindividual heterogeneity (Supplementary Table 3.2). These findings place *TERT* mutations as the most common genetic alteration in UBC[6, 7] and as an early event in urothelial carcinogenesis.

TERT mutations have been proposed to generate novel transcription factor binding sites leading to increased TERT expression.[12, 13] Mining the DNA-binding factor database JASPAR, we performed a comparative analysis of the wild-type and mutant sequences and found that the C228A mutation newly reported in this paper was also associated with an increased probability of binding to ELK1 (a member of the ETS oncogene family) and v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1), as the previously reported mutations. The analysis revealed additional transcription factors that could contribute to TERT overexpression as well some that showed mutation selectivity, such as v-rel avian reticuloendotheliosis viral oncogene homolog (REL) for C242T/C243T or GATA bind protein 2 (GATA2) for C228A (Supplementary Table 3.3). To assess the relationship between *TERT* mutations and mRNA levels, we analyzed 60 primary UBC samples using RT-qPCR. TERT mRNA levels were higher in *TERT* mutant tumors, but the difference was not statistically significant (Wilcoxon test $p = 0.3$). TERT mRNA levels were significantly higher in high-risk NMIBC and in MIBC than in low-risk NMIBC (Wilcoxon test $p = 0.001$) (Fig. 3.1D).

TERT mutations and patient outcome

The set of tumors from the Netherlands was used to assess tumor prognostic value. Of 184 patients with NMIBC, 90 (49%) experienced progression to MIBC (Table 3.1). There was no relationship between progression-free survival and the presence of mutations (log-rank test $p = 0.984$) (Supplementary Fig. 1). In the group of 173 patients with MIBC, 78 (45%) patients had local or distant progression and *TERT* mutation status was not associated with progression-free survival (log-rank test $p = 0.160$). Furthermore, 101 (58%) patients died during follow-up, including 65 (37%) who died of bladder cancer (Table 3.1). Overall- and cancer-specific survival were not related to *TERT* mutations (log-rank tests $p = 0.987$ and $p = 0.834$) (Supplementary Fig. 3.1). Among patients with MIBC, 25 received adjuvant chemotherapy with curative intent; *TERT* mutations were not predictive for survival in this subgroup or the group without chemotherapy.

TERT mutation detection in exfoliated cells in urine

Patients with NMIBC require continued follow-up due to frequent tumor recurrences. We assessed whether *TERT* mutations can be detected in exfoliated tumor cells in voided urine and compared the findings with *FGFR3* mutations (Table 3.3). Of 39 urine samples from patients with hematuria and in whom no bladder tumor was visible at cystoscopy (17 of 39 [44%] female; median age: 54 yr), 4 were positive for *TERT* mutations, resulting in a specificity of 90%. None of these four patients developed a UBC or another tumor in the subsequent >3.5 yr. In urine samples obtained before resection of a primary bladder tumor, the sensitivity of incident

tumor detection was 36% (46 of 128) for *FGFR3*, 62% (73 of 118) for *TERT*, and 70% (73 of 105) for *TERT* and *FGFR3* combined (*FGFR3* vs. *TERT*, $p < 0.0001$). We then tested 395 urine samples prospectively collected from 194 patients under surveillance after a primary NMIBC diagnosis. Sensitivity of detection of relapsing (recurrence or progression) UBC developing after a primary NMIBC was 19% (24 of 124) for *FGFR3*, 42% (48 of 113) for *TERT*, and 50% (52 of 103) for both combined (*FGFR3* vs. *TERT*, $p = 0.0001$). In the recurrence-free urine samples, a *TERT* mutation was detected in 58 of 218 cases, corresponding to a specificity of 73%, whereas the specificity for *FGFR3* mutation was 90% (*FGFR3* vs. *TERT*, $p < 0.0001$).

Table 3.3: Sensitivity and specificity of urine diagnostic assays

	Variable	The Netherlands, no./total (%)	Spain, no./total (%)	Netherlands and Spain, no./total (%)	Netherlands and Spain, *p* value
Primary tumors, sensitivity	*FGFR3*	17/42 (40)	29/86 (34)	46/128 (36)	–
	TERT	21/37 (57)	52/81 (64)	73/118 (62)	*FGFR3 vs TERT, p < 0.0001*
	TERT + FGFR3	20/28 (71)	53/77 (69)	73/105 (70)	*TERT vs TERT + FGFR3, p = 0.23*
Recurrent tumors, sensitivity	*FGFR3*	15/68 (22)	9/56 (16)	24/124 (19)	–
	TERT	18/63 (29)	30/50 (60)	48/113 (42)	*FGFR3 vs TERT, p = 0.0001*
	TERT + FGFR3	21/53 (40)	31/50 (62)	52/103 (50)	*TERT vs TERT + FGFR3, p = 0.238*
Recurrence-free FU urine samples, specificity	*FGFR3*	208/232 (90)	19/20 (95)	227/252 (90)	–
	TERT	149/200 (75)	11/18 (61)	160/218 (73)	*FGFR3 vs TERT, p < 0.0001*
	TERT + FGFR3	131/183 (72)	11/18 (61)	142/201 (71)	*TERT vs TERT + FGFR3, p = 0.529*

FGFR3 = fibroblast growth factor receptor 3; FU = follow-up; TERT = telomerase reverse transcriptase.

DISCUSSION

TERT promoter hotspot mutations constitute a new type of somatic genetic alterations in cancer. A recent survey in a wide range of tumors indicated a selective mutational pattern associated with tumor histology and tissue of origin.[14] It has been proposed that mutations occur more commonly in tumors derived from tissues with low proliferation rates.[12-14] Based on the analysis of 21 UBCs, a high mutation rate was proposed, but there was no clinical or pathologic information on these patients.

In this paper, we provide definitive evidence on the occurrence of *TERT* promoter mutations in UBC. First, we show a high mutation rate (70%), which was replicated in an independent series (79%) using different assays, thus making *TERT* the most frequently mutated gene in UBC.

Second, we show a similar mutation frequency across stages and grades, strongly suggesting that *TERT* mutations participate in the two major genetic pathways involved in UBC. Third, we show a distinct mutational spectrum in comparison with melanoma with a dominance of the C228T mutation. Finally, we show a lack of association with progression in NMIBC or with the development of local or distant metastases in MIBC.

The high frequency of hotspot mutations renders *TERT* a very attractive target for diagnosis of bladder tumors, both primary and recurrent, using body fluids such as urine or blood. We show that tumor cell detection in urine is feasible, with a sensitivity of 62% at initial diagnosis and 42% at recurrence. For urine diagnostics during surveillance, the patients were not stratified for mutation status of the primary tumor; we expect that when this is performed, sensitivity of detection of recurrent UBC will increase. Accordingly, *TERT* mutation was assayed concomitantly in tumor tissue and urine in 31 cases: *TERT* was concordant in 26 cases (19 mutated, 7 wild type), one tumor harbored a C242T mutation not assessable by the SNaPshot assay, and four mutations were detected in urine but not in tumor samples. We show that *TERT* mutation detection has a higher sensitivity than *FGFR3* mutation for tumor cell detection in urine, and assays of both *TERT* and *FGFR3* mutations might have improved sensitivity, though the difference was not statistically significant in our study. By contrast, *TERT* mutations may have a lower specificity than *FGFR3* mutations, as four mutations were detected in 39 urine samples from patients without cancer and in 58 of 218 of patients in clinical remission. In this group, we cannot distinguish reduced specificity from higher lead-time bias in detection of tumor relapse. Larger, well-designed, prospective studies are required to establish the clinical usefulness of mutation detection in urine. As previous reports have proposed TERT expression or activity assessment in urine as a tool to detect recurrence, it will be of high interest to compare *TERT* mutation with them and with other candidate biomarkers in future studies.[17, 18]

Several observations support the notion that *TERT* somatic mutations are an early event in urothelial carcinogenesis, including their occurrence in a small fraction of subjects without UBC, their presence in tumors of both the papillary and invasive pathways, and the low level of intraindividual heterogeneity when multiple tumor regions were analyzed. UBC cell lines are also very frequently mutated, and we noticed that most of the mutant ones lacked the wild-type allele. These observations also suggest that mutant cells may display a growth advantage over wild-type cells.

The mechanisms through which *TERT* promoter mutations contribute to UBC and other tumors remain to be determined. One possibility is that the acquisition of new, putative transcription binding sites leads to altered regulation of gene expression, as previously proposed, and also suggested by our bioinformatics analysis using the DNA-binding factor database JASPAR. However, we failed to find a significant association between *TERT* mutation and expression. This could be explained by alternative mechanisms of TERT upregulation in wild-type tumors, also leading to TERT overexpression, or by the participation of *TERT* mutations in additional biologic processes.

Given its reverse transcriptase activity, telomerase is a potential therapeutic target. Recent developments suggest that azidothymidine (AZT), an inhibitor of HIV reverse transcriptase, also inhibits telomerase. Hence, it is conceivable that AZT or other telomerase inhibitors may suppress the growth of UBC overexpressing the enzyme.[19]

One of the limitations of our work is the retrospective nature of the cohorts used for the outcome analysis, even though our results do not make positive claims. Another is the lack of comparison for urine-based diagnostic assays between *TERT* mutation, cytology, and other biomarkers. However, the high frequency of these mutations in urine reported herein provides evidence for future prospective and comparative studies. Overall, the novelty of *TERT* mutation in UBC is strengthened by the independent analysis that we performed in two different centers.

CONCLUSIONS

Mutations in the promoter of the *TERT* gene are the most frequent somatic mutations in tumors of the urinary bladder. Mutations occur with similar frequency, regardless of stage or grade, and are not associated with clinical outcome. *TERT* mutation assays may be used for tumor cell detection in urine and TERT may be a therapeutic target.

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SUPPLEMENTARY FILES

Reverse transcriptase quantitative polymerase chain reaction (PCR)

RNA was extracted using Trizol. Following DNAse treatment (DNAfree, Ambion; Invitrogen Corp, Carlsbad, CA, USA), complementary DNA was reverse transcribed (Taqman Reverse Transcription Reagents kit; Applied Biosystems, Foster City, CA, USA); 20 ng RNA equivalent was used for PCR with specific primers (AAGCATGCCAAGCTCTCG [forward], ATCAGCCAGTGCAGGAACTT [reverse]) in the presence of SYBR GreenER (Invitrogen Corp, Carlsbad, CA, USA) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All analyses were done in duplicate. A melting-curve analysis was performed to control for product quality and specificity. Levels of each transcript were normalized to individual hypoxanthine phosphoribosyltransferase 1 (HPRT) expression using the $\Delta\Delta$Ct method.

Bioinformatics analysis

The JASPAR CORE database[1] contains a curated, nonredundant set of profiles derived from published collections of experimentally defined transcription factor binding sites for eukaryotes. We scanned telomerase reverse transcriptase (*TERT*) mutations in a sequence of 20 nucleotides for the presence of the profiles included in the JASPAR CORE Vertebrata using the SCAN tool in JASPAR database Web server.[2] Relative profile scores increasing 0.05 point after including the mutation, when compared with the reference sequence, were predicted to have higher probability of binding. Scores decreasing 0.05 point were predicted to have lower probability of binding.

Supplementary Table 3.1: Results of telomerase reverse transcriptase (*TERT*) promoter mutational analysis in bladder cancer cell lines

Name	*TERT* promoter	Mutation status
92-1	1,295,228 G>A	Homozygous
96-1	1,295,228 G>A	Heterozygous
97-18	1,295,228 G>A	Homozygous
97-24	1,295,228 G>A	Homozygous
CRL-2742	1,295,228 G>A	Homozygous
HT1197	1,295,228 G>A	Homozygous
HU456	wt	
JON	wt	
KK47	wt	
LGWO 1 G600	wt	
MG-HU-3	1,295,228 G>A	Homozygous
MG-HU-4	1,295,250 G>A	Heterozygous
PSI	1,295,228 G>A	Homozygous
RT112	1,295,228 G>A	Heterozygous
RT4	1,295,228 G>A	Homozygous
SCaBER	1,295,228 G>A	Homozygous
SW1710	1,295,228 G>A	Homozygous
SW780	1,295,228 G>A	Homozygous
SW800	1,295,228 G>A	Homozygous
T24	1,295,228 G>A	Homozygous
TCC-SUP	1,295,228 G>A	Homozygous
UM-UC-10	1,295,228 G>A	Homozygous
UM-UC-11	1,295,250 G>A	Homozygous
UM-UC-13	1,295,228 G>A	Homozygous
UM-UC-14	1,295,228 G>A	Heterozygous
UM-UC-15	1,295,228 G>A	Heterozygous
UM-UC-18	1,295,228 G>A	Homozygous
UM-UC-2	1,295,228 G>A	Homozygous
UM-UC-5	1,295,228 G>A	Homozygous
UM-UC-7	1,295,228 G>A	Heterozygous
UM-UC-9	1,295,250 G>A	Homozygous
VM-CUB-1	1,295,228 G>A	Homozygous

Supplementary Table 3.2: Telomerase reverse transcriptase (*TERT*) mutations assessed in distinct regions of non–muscle-invasive bladder cancer (NMIBC)

Case NMIBC	Fragment	*TERT* promoter
IS_11453	#1	1,295,250 G>A
	#2	1,295,250 G>A
	#3	1,295,250 G>A
	#4	1,295,250 G>A
	#5	1,295,228 G>A
IS_11545	#1	1,295,228 G>A
	#2	1,295,250 G>A
	#3	wt
	#4	1,295,228 G>A
IS_11656	#1	wt
	#2	wt
IS_11801	#1	1,295,250 G>A
	#2	1,295,250 G>A
	#3	1,295,250 G>A
	#4	1,295,250 G>A
IS_11818	#1	1,295,228 G>A
	#2	1,295,228 G>A
	#3	1,295,228 G>A
	#4	1,295,228 G>A
IS_11825	#1	1,295,228 G>A
	#2	wt
	#3	1,295,228 G>A
IS_11832	#1	wt
	#2	wt
	#3	wt
	#4	wt
IS_11834	#1	1,295,228 G>A
	#2	1,295,228 G>A
	#3	wt
IS_11837	#1	wt
	#2	wt

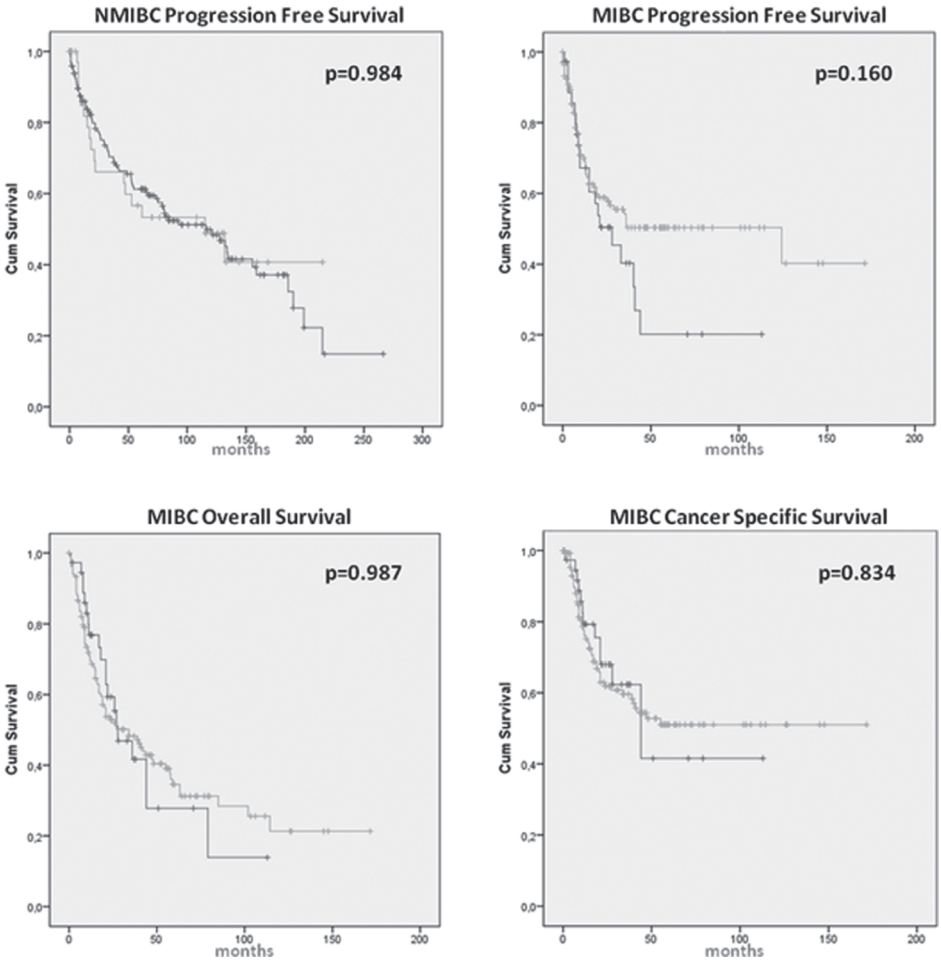
Supplementary Table 3.3: Bioinformatic analyses of sequences around the hotspot mutations using JASPAR

Genomic coordinate		>chr5:1295218-1295238	>chr5:1295218-1295238	>chr5:1295218-1295238			
Sequence		gggcccggagggg gctgggc	gggcccggaaggg gctgggc	gggcccggatggg gctgggc			
		G228G	**G228A ***	**G228T ***			
Model ID	Model name	Relative score	Relative score	Relative score	Start	End	Strand
MA0098.1	ETS1	0.82	**1.00**	**0.98**	6	11	-1
MA0028.1	ELK1	0.81	**0.94**	**0.85**	2	11	1
MA0062.2	GABPA	0.81	**0.93**	0.82	5	15	1
MA0145.1	Tcfcp2l1	0.90	0.91	0.95	5	18	-1
MA0003.1	TFAP2A	0.98	0.89	**0.87**	3	11	1
MA0079.2	SP1	0.94	0.87	0.87	10	19	-1
MA0144.1	Stat3	0.72	**0.82**	0.71	2	11	1
MA0136.1	ELF5	0.68	**0.81**	**0.78**	5	13	-1
MA0076.1	ELK4	0.70	**0.81**	0.76	4	12	1
MA0103.1	ZEB1	0.65	**0.81**	0.62	9	14	-1
MA0156.1	FEV	0.67	**0.81**	0.71	5	12	1
MA0081.1	SPIB	0.64	**0.80**	0.68	4	10	1
MA0057.1	MZF1_5-13	0.84	0.80	0.81	7	16	1
MA0116.1	Zfp423	0.79	0.80	0.72	1	15	1
MA0163.1	PLAG1	0.81	0.77	0.77		14	1
MA0056.1	MZF1_1-4	0.78	0.76	0.81	10	15	1
MA0095.1	YY1	0.69	0.69	**0.90**	8	13	-1
MA0036.1	GATA2	0.68	0.68	**0.96**	7	11	1

Genomic coordinate		>chr5:1295232-1295252	>chr5:1295232-1295252			
Sequence		ctgggccggggacccgggag	ctgggccggaaacccgggag			
		GG242GG	**GG242AA**			
Model ID	Model name	Relative score	Relative score	Start	End	Strand
MA0098.1	ETS1	0.63	**1.00**	7	12	-1
MA0028.1	ELK1	0.84	**0.98**	11	20	1
MA0062.2	GABPA	0.63	**0.87**	6	16	1
MA0101.1	REL	0.62	**0.87**	8	17	-1
MA0003.1	TFAP2A	0.89	0.86	5	13	1
MA0076.1	ELK4	0.63	**0.86**	5	13	1
MA0133.1	BRCA1	0.60	**0.83**	9	15	1
MA0107.1	RELA	0.64	**0.82**	8	17	-1
MA0156.1	FEV	0.54	**0.81**	6	13	1
MA0081.1	SPIB	0.48	**0.80**	5	11	1
MA0039.2	Klf4	0.82	0.74	2	11	1
MA0146.1	Zfx	0.82	0.74	2	15	-1
MA0079.2	SP1	0.84	**0.70**	2	11	-1
MA0039.2	Klf4	0.82	**0.67**	3	12	1
MA0056.1	MZF1_1-4	0.97	**0.59**	7	12	1

Genomic coordinate		>chr5:1295240-1295260	>chr5:1295240-1295260			
Sequence		gggacccgggaggggtcggg	gggacccggaaggggtcggg			
		G250G	**G250A**			
Model ID	Model name	Relative score	Relative score	Start	End	Strand
MA0098.1	ETS1	0.82	**1.00**	7	12	-1
MA0028.1	ELK1	0.84	**0.96**	3	12	1
MA0062.2	GABPA	0.80	**0.92**	6	16	1
MA0155.1	INSM1	0.77	0.83	7	18	1
MA0136.1	ELF5	0.68	**0.81**	6	14	-1
MA0076.1	ELK4	0.69	**0.81**	5	13	1
MA0103.1	ZEB1	0.71	**0.81**	10	15	-1
MA0156.1	FEV	0.67	**0.81**	6	13	1
MA0081.1	SPIB	0.64	**0.80**	5	11	1
MA0057.1	MZF1_5-13	0.84	0.79	9	18	1
MA0079.2	SP1	0.82	0.77	6	15	-1
MA0039.2	Klf4	0.85	**0.73**	7	16	1

** Situations in which increased binding to a specific transcription factor is predicted in the mutated sequence are screened in dark grey. Situations in which reduced binding is predicted are screened in light grey.*



Supplementary figure 3.1 *Actuarial survival curves according to telomerase reverse transcriptase (TERT) mutation: progression-free survivals in non–muscle-invasive bladder cancer (NMIBC) and MIBC, and overall and cancer-specific survival in MIBC. Cum = cumulative.*

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PART III

URINARY BIOMARKERS FOR THE DETECTION OF BLADDER CANCER

Chapter 4

A methylation assay for the detection of non-muscle-invasive bladder cancer recurrences in voided urine

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ABSTRACT

Objective | To develop a Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay for the detection of non-muscle invasive bladder cancer (NMIBC) recurrences in voided urine.

Material and methods | Genes frequently methylated in NMIBC tumors (n=37) were selected to develop a BC-specific MS-MLPA assay and genes methylated in urine from non-BC patients (n=46) and blood from BC patients (n=29) were excluded. A four-gene panel with the highest predictive value was selected from the initial assay. This four-gene panel was tested and validated on urine of patients with a histologically proven recurrence (n=68 test set; n=49 validation set) and urine samples from non-BC patients (n=91 test set) and urines from recurrence-free BC (rec-free BC) patients (n=60). A model was developed to predict the probability of having a recurrence based on methylation of the four-gene panel and a cut-off probability with the highest sensitivity and specificity was determined. Outcome of the model was validated on BC urine samples (n=65) and on urine samples from rec-free BC patients (n=29).

Results | The BC MS-MLPA assay consisted of 23 methylation probes. The selected four-gene panel included: *APC*, 2x *hTERT*, and *EDNRB*. This panel reached an area under the ROC curve (AUC) of 0.82 (test set) and AUC: 0.69 (validation set). Sensitivity and specificity for the detection of a concomitant tumor were 63.3% and 58.3% respectively (test set) and 72.3% and 55.2%, respectively (validation set).

Conclusions | We have developed a methylation detection assay specifically for the detection of recurrences of NMIBC patients on voided urine. The findings are promising and improvement of this test could eventually contribute to a more individualized patient friendly surveillance.

INTRODUCTION

Bladder cancer (BC) is the most prevalent type of urothelial cell carcinoma and is associated with the highest costs in patient surveillance compared to other types of cancer.[1] This is mainly caused by the high recurrence rate of non-muscle invasive bladder cancer (NMIBC), which necessitates life-long cystoscopic follow-up and frequent trans-urethral resections (TUR).[2-4] Today, cystoscopy remains the golden standard for surveillance of NMIBC patients, but this is a costly and time-consuming procedure with a sensitivity ranging from 68-83% and causing physical discomfort to the patient.[5-8] During follow-up, cytology is used in addition to cystoscopy, however the sensitivity of cytology is specifically insufficient for the detection of low stage and grade tumors, whereby its accuracy is influenced by the pathologist's experience.[9-14] Many urinary biomarkers have been identified to reduce the number of cystoscopies and BC-associated costs, hereby improving patient quality of life and patient care. Most available biomarkers have a low sensitivity for the detection of NMIBC recurrences, hence the clinical applicability of these markers remains limited. Together, these findings emphasize the need for identification of new urinary biomarkers for the follow-up of NMIBC patients.

Recently, the role of epigenetic changes in BC has become more apparent. Epigenetic changes are defined as changes in gene expression that are heritable through cell division, without associated DNA sequence alterations. Previous studies demonstrated the role of aberrant DNA promoter hypermethylation, leading to transcriptional silencing of tumor suppressor genes.[15-17] Hypermethylation in BC has been associated with age, smoking status, gender, tumor location, stage, recurrence rate and progression.[18-22] In addition to detection of methylation in tumor tissue, aberrant methylation has also been detected in voided urine of BC patients and appears to be more sensitive than urine cytology.[23-30]

Current urinary biomarkers are mostly tested and validated on primary tumors, which are large in size and thus easier to detect in urine than in recurrent tumors. In addition to this, muscle-invasive (MI) tumors are used to validate markers for the detection of recurrent bladder tumors. MI tumors are also larger in size, are high grade with more genetic aberrations and shed more tumor cells than NMI tumors. Thus, they are easier to detect by urine-based assays, not reflecting the true sensitivity of the assay for the detection of NMIBC recurrences. Therefore, we designed a Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay for the detection of genes specifically methylated in recurrences of NMIBC patients. We selected a smaller gene panel from the BC MS-MLPA assay and developed a logistic regression model to predict the recurrence probability based on genes methylated in urinary tumor cells. Lastly, we determined the sensitivity and specificity of the selected gene panel on an independent test and validation cohort.

MATERIAL AND METHODS

The study design consisted of a four step process to develop the MS-MLPA specifically for the detection of NMIBC recurrent tumors (Figure 4.1, Table 4.1). The Medical Ethical Committee of the Erasmus MC approved the study (MEC 168.922/1998/55) and written informed consent was obtained from all patients.

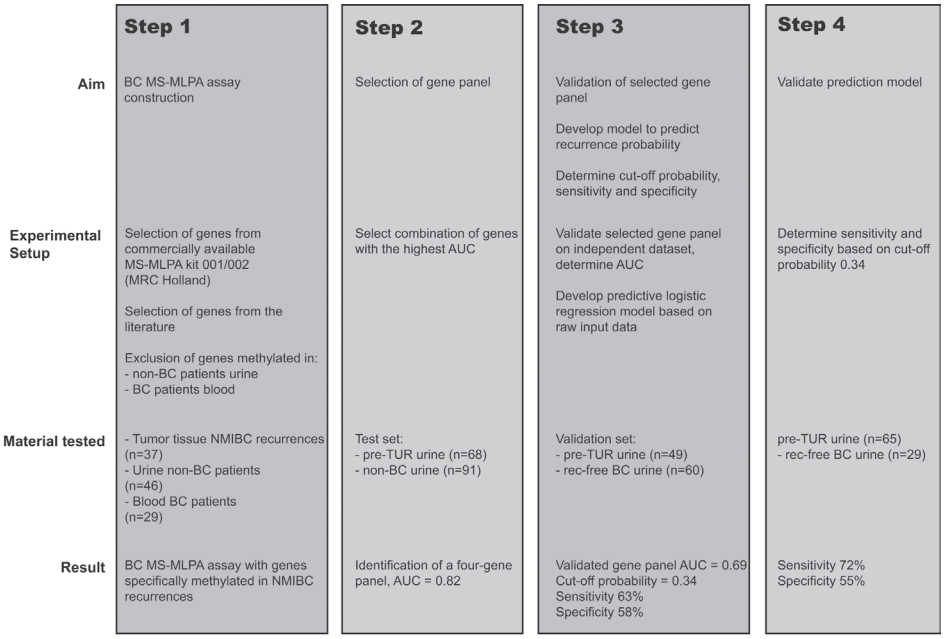

Figure 4.1 *Four-step process to select genes that are specifically methylated in NMIBC recurrences, select a smaller gene panel, validate this gene panel and develop a prediction model for the detection of BC recurrences in voided urine. Non-BC: non-bladder cancer patients; rec-free BC; recurrence-free BC patients; Pre-TUR: urine collected 1 day before transurethral resection of tumor*

Study population

Step 1. Recurrent tumors from patients included with a primary NMI bladder tumor were selected (n=37) for methylation analysis. As a control group, urine from patients with benign urological disorders other than BC (non-BC) (n=46) was collected prior to cystoscopy at the Erasmus MC Urology outpatient department and stored at 4ºC. All patients with a history of urinary tract malignancies (bladder, upper urinary tract, prostate and kidney) were excluded. Patients with benign prostate hyperplasia, which is age related and often diagnosed in elderly men, were included in the control group. The reason for inclusion is the concurrent presentation with bladder cancer, also developing in elderly men. Samples were checked for leucocytes, erythrocytes and nitrite (Bayer, multistix 10 SG). Additionally, 29 blood samples from BC patients were included in the study.

Step 2. Urine samples collected one day before TUR of the recurrent tumor (pre-TUR urine) (n=68) were selected for analysis. After TUR, stage and grade of the recurrent tumor were confirmed by an expert uro-pathologist. Control urine samples from non-BC patients (n=91) were drawn from the Dutch bladder cancer screening study.

Step 3. Urine samples from BC patients collected during follow up (n=109) were used for analysis. From this group 49 patients had a histologically proven recurrence at the time of urine collection. The other 60 urines were from BC patients who were recurrence-free at the time of urine collection.

Step 4. Urine samples from an independent set of BC patients (n=94) were used to validate the developed logistic regression model. From this group 65 urines were from patients with a histologically proven recurrent tumor at the time of urine collection. The other 29 urine samples were from BC patients who were recurrence-free at the time of urine collection.

Table 4.1: Patient and tumor characteristics

	Age (years)		Tumor stage, n(%)					Tumor grade, n(%)			
Characteristics	N	Mean (SE, range)	N	Ta	T1	T2	Tis	G0	G1	G2	G3
Step 1											
Tumor	37	69 (15, 26-86)	37	36 (97)	1(3)	-	-	-	14 (38)	23 (62)	-
Non-BC urine	46	54 (14, 26-74)									
Blood	29	59 (10, 40-80)									
Step 2											
BC- urine	68	65 (11, 35-85)	68	61(90)	7 (10)	-	-	-	14 (31)	52 (77)	3 (4)
Non-BC urine	91	62 (6, 51-73)									
Step 3											
Follow-up urine	109	63 (12, 20-82)	49	38 (78)	9 (18)	-	2(4)	-	15 (31)	22 (45)	12 (24)
Step 4											
Follow-up urine	94	64 (11, 42-86)	65	58 (89)	6 (9)	1 (2)	-	3 (5)	25 (38)	29 (45)	8 (12)

Non-BC urine = urine from patients without BC; BC urine = urine from patients with concomittant BC; Follow-up urine = urine collected from patients with BC during follow-up with and without recurrence

DNA isolation from patient material

Fresh tumor tissue: Tumor tissue was collected at a TUR at Erasmus Medical Center, Rotterdam. Part of the tissue was used for DNA isolation and the other part was used for a histological report. Muscle-invasive tumors and carcinoma in situ were excluded from analysis. Fresh tumor samples for DNA isolation were stored at -80°C until DNA isolation. DNA was extracted using the Qiagen Dneasy blood and tissue kit (Qiagen, GmbH, Hilden Germany) according to the manufacturer's protocol and analyzed with the MS-MLPA assay.

Urine: Freshly voided urine samples (10-100ml) were centrifuged for 10 minutes' at 3000 rpm. Cell pellets were washed twice with 10ml of Phosphate-buffered saline, followed by centrifugation for 10 minutes' at 3000 rpm. Pellets were resuspended in 1ml of Phosphate-buffered

saline, transferred to an eppendorf vial and centrifuged for 5 minutes' at 6000 rpm (3000xg). Supernatant was discarded and the cell pellet was stored at -20ºC until DNA isolation. DNA was extracted using the QiAamp Mini and Blood mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Blood: Freshly extracted blood samples were stored at -20°C until DNA isolation. DNA was extracted using the QIAamp DNA blood mini kit (Qiagen, GmbH, Hilden Germany) according to the manufacturer's protocol.

Methylation-specific multiplex ligation probe amplification assay

50 ng of fresh tumor tissue or urine-derived genomic DNA was used and MS-MLPA assays were performed as described by van Schouten *et al*[31]. Briefly, the MS-MLPA technique (MRC Holland) is based on DNA sample denaturation followed by hybridization of probes directed to one specific CpG site that contains a restriction site for the methylation sensitive endonuclease HhaI enzyme. Following probe hybridization and ligation unmethylated hybrids are digested by HhaI and will not be exponentially amplified by PCR, leading to the absence of a signal when analyzed by capillary electrophoresis. In contrast, methylated hybrids are prevented from being digested by HhaI and ligated probes will generate a signal after amplification by PCR. The MS-MLPA contains 18 reference probes that do not contain a HhaI restriction site, and thus are not sensitive to HhaI digestion.

MRC kit-001 contains probes for 26 tumor suppressor genes with 15 reference probes and kit-002 contains 27 probes for tumor suppressor genes with 14 reference probes. Kit-001 and 002 contain probes for the same genes, but the probes recognize a different CpG-site.

Statistical analyses

Statistical analysis for probe selection of the BC MS-MLPA kit was performed using the Statistical Package for Social Sciences 11.5 (SPSS Inc, Chicago) and R statistical Software (R Foundation for Statistical Computing, Vienna). The probes were selected with the LASSO (least absolute shrinkage and selection operator) method. This method combines selection and estimation by shrinking coefficients, some effectively to zero. The purpose of using the LASSO method was to prevent overfitting, i.e. probes being selected by coincidence. Consecutively the probes with non-zero coefficients in the LASSO were combined into a logistic regression model to determine their combined area under the curve.

RESULTS

Step 1. Construction of the BC MS-MLPA

In order to select genes for a bladder cancer specific MS-MLPA assay, recurrent NMIBC tumors (n=37) were analyzed with the commercially available MS-MLPA kit 001 and 002 and methylated genes were selected to develop a BC specific MS-MLPA (Figure 4.1, Step 1). Urine samples

of non-BC patients (n=46) and blood samples from NMIBC patients (n=29) were selected as controls. Patient and tumor characteristics are depicted in Table 4.1 (Step 1). Methylation of *TIMP3* (p=0.022), *APC* (p=0.004), *RARB* (p=0.022), *DAPK* (p=0.022), and *ESR1* (p=0.001) from kit-001 was significantly higher in BC tumor tissue compared to urine of non-BC patients. Methylation of *TP53* (p=0.034), *MGMT* (p=0.031), *PAX5* (p<0.0001), *CDH13* (p<0.0001), *TP73* (p=0.039), *WT1* (p<0.0001), *ESR1* (p<0.0001), *MSH6* (p=0.005), *RARB* (p=0.005), and *CD44* (p=0.039) from kit-002 was significantly higher in BC tumor tissue compared to urine of non-BC patients. Interestingly, *RASSF1A* (kit-001) was methylated in 53% of the non-BC urine samples. Genes methylated in urine from non-BC patients and genes methylated in blood were excluded. The selection of genes for the BC MS-MLPA was based on above findings with the addition of 6

Table 4.2: Bladder cancer probe mix

Length (nt)	Gene	Partial sequence with HhaI site
64-70-76-82		DNA quantity control fragments
88-92-96		Denaturation, hybridization and ligation control fragments
124	TRAF4	TGCCAAGGTGAGTCCACACTGCCAGGA-AGAAGCCCAAGCACAGTCTGTTGCCCTTGGA
130	BCL2	TGTGAGATGTCCAGCCAGCTGCACCTG-ACGCCCTTCACCGCGCGGGGACGCTTTGCCA
136	PTEN	GCTCTTTCATTTTTAGGGCAAACGAGCCGAGT-TACCGGGGAAGCGAGAGGTGGGGCGCTGCAAG
142	TIMP3	TCCAGCGCCGAGGCAGCCTCGC-TGCGCCCCATCCCGTCCCGCCGGGCACTCGG
148	APC	GGCTGGGTGTGGGCGCACGT-GACCGACATGTGGCTGTATTGGTGCAGCCCGCCAG GGT
154	TERT	CGCAGCGCGGGGACCCGGCGGCTT-TCCGCGCGCTGGTGGCCCAGTGCCTGGT
160	TERT	TCTGTGTCCTGCCTGAAGGAGCTGGT-GGCCCGAGTGCTGCAGAGGCTGTGCGAGCGCG
172	WIF1	TCTGTCTAAACGGGAACAGCCCTGGCT-GAGGGAGCTGCAGCGCAGCAGAGTATCTG ACGGCGCC
178	CCM2	CTGGAATTGTCTCGCCATTTAAACGAGTAT-TCCTAAAAGGTGAAAAGAGTAGAGATAAG AAAGCCCATGAGAAGGT
193	RARB	CCGCCGGCTTGTGCGCTCGCT-GCCTGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCT
202	MLH3	GCGACCTTGTTCTTCCTTTCCTTCCGA-GAGCTCGAGCAGAGAGGACTGTGATGAGAC AGGATAACAG
211	TGFBR1	GGCGTTACAGTGTTTCTGCCACCTCT-GTACAAAAGACAATTTTACTTGTGTGACAGATG GGCTCTGCTTT
220	COL2A1	GCCTCTTCCTTCTACACAGGGTCCTT-CTGGAGACCAAGGTGCTTCTGGTCCTGCTGGT CCTTCT
229	PAH	CAGTGCCCTGGTTCCCAAGAA-CCATTCAAGAGCTGGACAGATTTGCCAATCAGATTC TCAG
238	CDKN2A	CTGGATCGGCCTCCGACCGTAAC-TATTCGGTGCGTTGGGCAGCGCCCCCGCCTCCA GCAGC
247	PTCH	GTGGACAGCTGGGAGGAAATGCTGAA-TAAGGCTGAGGTTGGTCATGGTTACATGGA CCGCCCCT

Table 4.2: Bladder cancer probe mix (continued)

Length (nt)	Gene	Partial sequence with HhaI site
259	CDKN2A	CCAGGGGCACCAGAGGCAGTAACCATG-CCCGCATAGATGCCGCGGAAGGTCCCTCAGA
265	SLC2A1	CTCTGGTCCCTCTCAGTGGCCATCTTTTCT-GTTGGGGGCATGATTGGCTCCTTCTCTGTG GGCCTTTTCGTTAACC
283	BCL2	GTGAAGCGGTCCCGTGGATAGAGA-TTCATGCCTGTGCCCGCGCGTGTGTGCGCGCG TG
292	PTEN	CACCGGAGCGGGCGCAGGAGA-GGCCTGCGGGGTGCGTCCCACTCACAGGGAT
301	ESR1	GCTCGCGTGTCGGCGGGACAT-GCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTT TTCC
310	DBC1	CTGGAGGTTTGTTGAGCTCCTCTACT-TCCTGTTTATATGGGGCCGTATCTCAGTGCAGC CC
319	TIMP3	CATCGTGCTCCTGGGCAGCTGGA-GCCTGGGGGACTGGGGCGCCGAGGCGTGCACA
328	KRIT1	TGGTCTAGGAGCTCCAGACTACTCAAAAAT-CCAAATACCTAAACAGGAAAAATGGCAG AGAAGCATGAGCAGT
337	CDH1	CTATGAAGGAAGCGGTTCCGAAGCTGCTA-GTCTGAGCTCCCTGAACTCCTCAGAGTC AGACAAAGACCAGGAC
346	TNFRSF25	CTGAAGGCGGAACCACGACGGGCA-GAGAGCACGGAGCCGGGAAGCCCCTGGGC GCCCG
355	TGFBR1	GAGAATGTTGGTATGCCAATGGAGCA-GCTAGGCTTACAGCATTGCGGATTAAGAAAA CATTATCGCA
364	DBC1	TCACTTCCTGACCATCGGAAGCATCCA-TCAGCACTGGGGCAATGACTGGGACCTGCAGA
375	ESR1	CCAGCCCGCCGTGTACAACTACCCCG-AGGGCGCCGCCTACGAGTTCAACGCCGCGGC
382	PTCH	GATAAGAGCTCCGGGGGGATTCTTCA-TGCACCAGTGTTTAGGGTACGTGCTTCCTAAG TAAATCCAAACATTG
391	CDKN2A	GCTCTTCCGCCAGCACCGGAGGAA-GAAAGAGGAGGGGCTGGCTGGTCACCAGAG GGTG
400	TIMP3	CCAGCGCTATATCACTCGGCCGCCCA-GGCAGCGGCGCAGAGCGGGCAGCAGGCAG
409	EDNRB	CCAAGTTTCCCACTGGCGCGCAA-ACTTGAGTTACTTTTGAGCGTGGATACTGGCGAA GAGGCTG
418	TERT	CCAGAGTTTCAGGCAGCGCTGCGTCCT-GCTGCGCACGTGGGAAGCCCTGGCCCCGGCC
427	APAF1	CCATTCATGCTTGTTTTGTTTTGGATTTTAGTAAGGACA-GTCCTGTGTGAAGGTGGAGTA CCACAGAGGCCAGTTGTTTTTG
436	CDH13	GTTCTGTGCGTTCTCCTGTCCCAG-GTAGGGAAGAGGGGCTGCCGGGCGCGCTCTG
445	DBC1	GCAGTCACTCCTCTACTGTAATGAGAATGGGTT-TTGGGGAACCTTCCTGGAGAGCCAG CGGAGCTGCGTG
454	APC	CTCAGCTGTGTAATCCGCTGGATGCGGACC-AGGGCGCTCCCCATTCCCGTCGGGAGCCCGC
463	WIF1	CCTGCAAATAGAGCGAGAACAGAAGAGCGGGA-AGGGCTGGCGCGAGCGAGGTGCGAGCGAGGAG
472	CDH1	CTGAGGAGCGGAGCGGCCTGGAA-GCCTCGCGCGCTCCGGACCCCCCAGTGATGG GAGT
481	RARB	CCACATGTGCTTTTTCTGGAGTGGAAAAATACATA-AGTTATAAGGAATTTAACAGACAG AAAGGCGCACAGAGGAATTT

genes methylated in BC patients selected from the literature[18, 19, 25, 28, 32]. Due to space restraints selected genes from kit-001 were: *TIMP3*, *APC*, *RARB*, *PTEN*, and *CDH13*. *ESR1* was selected from kit-002. Genes selected from the literature included *BCL2*, *HTERT*, *TNFRSF25*, *EDNRB*, *CDH1* and *WIF1*. An overview of the final BC MS-MLPA containing 23 methylation probes is demonstrated in Table 4.2. It should be noted that the BC MS-MLPA assay contains multiple probes for *BCl2, PTEN, TIMP3, APC, TERT, WIF1, RARB, ESR1* and *CDH1*. Next, we analyzed the same available tumors and control non-BC urine samples with the newly developed BC MS-MLPA to validate the selection of probes. Results from the analyzed non-BC urine samples (n=40) and NMIBC tumor samples (n=34) are displayed in Table 4.3 Significantly higher methylation of tumor tissue was reached for *TERT_b* (p<0.0001), *ESR1_a* (p<0.0001), *EDNRB* (p<0.0001), *TERT_c* (p=0.001) and *CDH13* (p<0.0001). Methylation of *TNFRSF25* was significantly higher in the non-BC control group. In summary the newly designed BC custom MS-MLPA contains gene probes specifically methylated in tumor tissue of NMIBC patients.

Table 4.3: Methylation frequencies for BC Ms-MLPA custom kit

	Urine non-BC (n=40)		Tumor tissue (n=34)		
Genes	M	%M	M	%M	p- value
BCL2_a	5	12,5	13	38,2	0,01
PTEN_a	0	0	1	2,9	0,459
TIMP3_a	0	0	1	2,9	0,459
APC_a	0	0	6	17,6	0,007
TERT_a	0	0	6	17,6	0,007
TERT_b*	0	0	10	29,4	<0.0001
WIF1_a	0	0	2	5,9	0,208
RARB_a	0	0	5	14,7	0,017
CDKN2A	0	0	1	2,9	0,459
BCL2_b	0	0	1	2,9	0,459
PTEN_b	0	0	1	2,9	0,459
ESR1_a*	0	0	27	79,4	<0.0001
TIMP3_b	0	0	1	2,9	0,459
TNFRSF25	35	87,5	31	91,2	0,719
ESR1_b	0	0	2	5,9	0,208
TIMP3_c	0	0	1	2,9	0,459
EDNRB*	4	10	23	67,6	<0.0001
TERT_c*	0	0	8	23,5	0,001
CDH13*	0	0	9	26,5	<0.0001
APC_b	0	0	6	17,6	0,007
WIF1_b	5	12,5	14	41,2	0,005
CDH1	0	0	1	2,9	0,459
RARB_b	1	2,5	3	8,6	0,328

**Statistical significant methylated genes. Chi-square test (Bonferroni correction α=0,002)*

Step 2. A four-gene panel for the detection of recurrences

We aimed at selecting a smaller gene panel to improve the utility of the developed BC MS-MLPA. (Figure 4.1, Step 2). To this end, the LASSO-method was used to select a combination of methylated gene probes with the highest predictive value, based on a dataset containing 68 pre-TUR urine samples versus urine samples from non-BC patients (n=91). The gene probes *APC_a*, *TERT_a*, *TERT_b* and *EDNRB* were identified by the LASSO approach as discriminating between tumor and control and were combined into the final gene panel. This panel reached an AUC under the ROC curve of 0.82 (Figure 4.2A).

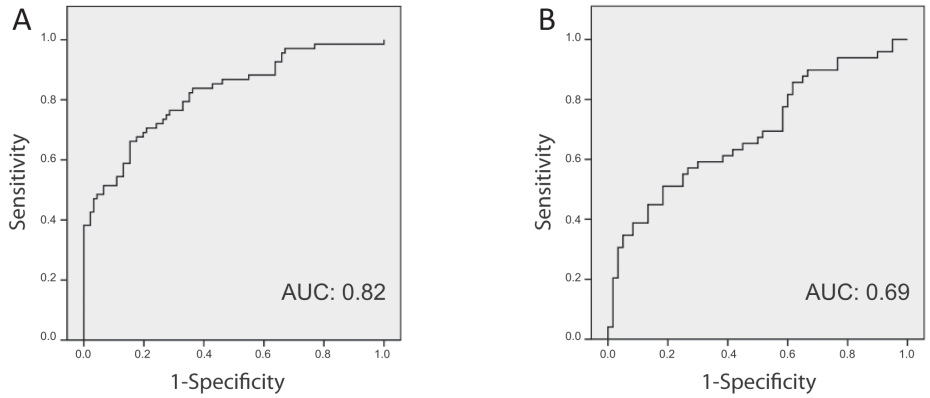

Figure 4.2 *ROC and AUC of the four-gene panel. (**A**) ROC of gene panel: APC a , TERT a , TERT b and EDNRB for the test set (AUC 0.82) and (**B**) validation set (0.69).*

Step 3. Recurrence risk probability increases with stage and grade

The selected gene panel was validated on an independent dataset of 49 pre-TUR urines and 60 control urines of recurrence-free BC patients. The AUC for the gene panel reached 0.69 in this validation (Figure 2B). Next, we developed a logistic regression model, based on methylation of the gene panel, to predict the probability of a recurrence: (APC_a *0.097) + (Tert_a *0.507) + (Tert_b * 0.218) + (EDNRB *0.501) + -2.480. Raw methylation outcomes were used as input in the logistic regression model to predict the recurrence probability, without converting results to a dichotomized variable. This was done to prevent loss of any data. A probability cut-off point (0.34), with the most optimal sensitivity and specificity, was selected to indicate whether a test outcome was considered positive (detected recurrence) or negative (missed recurrence) (Figure 4.3A). This resulted in a sensitivity and specificity of 31/49 (63.3%) and 35/69 (58.3%), respectively (Table 4.4). All high grade tumors i.e. T1, grade 3 and carcinoma in situ, were detected and most were associated with a high recurrence risk probability. Missed tumors were of low risk potential, namely TaG1-2.

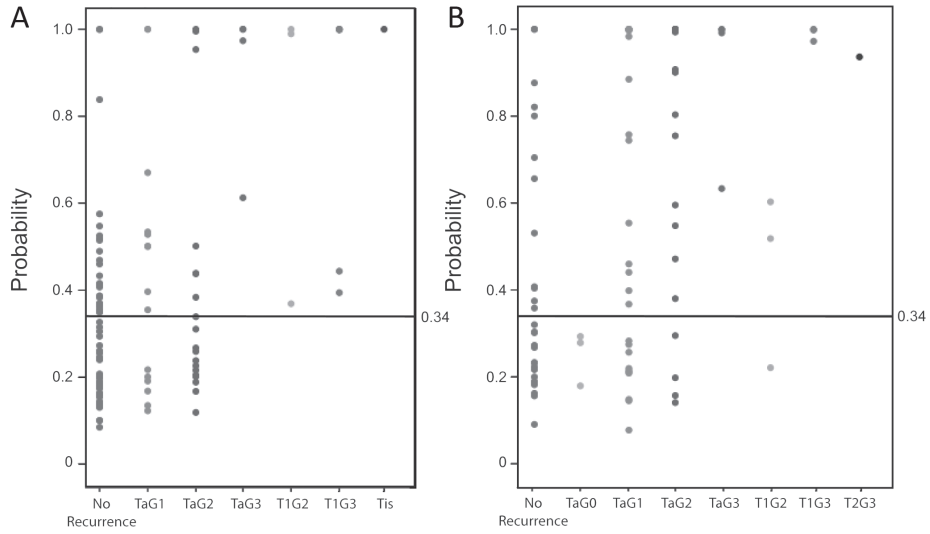

Figure 4.3 Probability plot for the detection of a recurrent tumour in voided urine. Probability plot based on the four-gene panel. X-axis depicts stage and grade of the resected recurrences. Y-axis depicts the predicted recurrence probability based on the developed logistic regression model. Test outcome was considered positive (detected recurrence) based on the probability outcome > 0.34 and negative (missed recurrence) < 0.34. Each dot represents a urine sample.

Table 4.4: Sensitivity and specificity of the four-gene panel in the test and validation set of urine samples

	Test set	Validation set
Sensitivity rate of gene panel, methylated (>0.34)/ total (%)		
All cancer	31/49 (63)	47/65 (72)
Ta	20/38 (53)	41/58 (71)
T1	9/9 (100)	5/6 (83)
Tis	2/2 (100)	1/1 (100)
Low grade	19/37 (51)	39/57 (68)
High grade	12/12 (100)	8/8 (100)
Specificity rate of gene panel, not methylated (<0.34)/total (%)		
Recurrence-free patients without BC	35/60 (58)	16/29 (55)

Step 4. Methylation-based probability, a promising tool for the detection of high risk tumors

Finally, we validated the selected cut-off probability of 0.34 on an independent set of 65 pre-TUR urine samples and 29 urine samples from recurrence-free BC patients. The sensitivity and specificity were 47/65 (72.3%) and 16/29 (55.2%), respectively (Table 4.4). High risk tumors were detected in 91% (10/11). Mostly pTaG1-2 tumors were missed, together with one pT1G2 tumor was missed (Figure 4.3B).

DISCUSSION

Promoter methylation is a frequent finding in bladder tumors and previous research has indicated the possibility of methylation detection in urine. Since methylation has been associated with bladder tumor stage, grade, recurrence and progression detection of methylated tumor cells shed in urine might be a promising tool during the follow-up of NMIBC patients.[18] We developed an MS-MLPA assay specifically for the detection of NMIBC recurrences and selected a smaller panel of four probes from the original MS-MLPA to improve utility of the assay. Next, we developed a logistic regression model based on this panel to determine the probability of having a recurrence and found that the model was able to predict recurrent tumors and that the recurrence risk increased with tumor stage and grade.

Since voided urine does not contain many cells and there is a limited availability of clinical samples for molecular analyses, newly developed tests should be able to detect a low number of tumor cells and require small amounts of input DNA. The BC MS-MLPA assay is a sensitive method for detecting tumor cells and requires only 50ng of urinary DNA input.[31] The assay is based on a multiplex PCR to investigate the methylation status of up to 23 genes in one assay, hereby enabling simultaneous analysis of multiple patient samples without an increase in associated costs and the use of large amounts of DNA. Futhermore, the assay does not require bisulfite conversion, which is known to cause DNA breakage and thus might affect the sensitivity of a test.

The sensitivity for detection of recurrences in the test and validation set were 63% and 72% respectively. Interestingly, all high-grade recurrences (including one T2G3) were detected in the test and validation set and mostly associated with a high risk probability. Most of the undetected tumors were low risk TaG1-2. Only one pT1G2 was missed. Thus, the methylation detection assay is able to detect recurrent and progressing tumors. Compared to previous studies the sensitivity of this assay is relatively low.[25, 27, 28, 33] Friedrich *et al.* reported a sensitivity of 78% for the detection of methylation in urine samples associated with a primary tumor collected before radical cystectomy (22/37 ≥pT2).[25] Although size was not recorded in their manuscript, primary tumors and tumors selected for radical cystectomy are often large in size, have a higher grade and more genetic aberrations than recurrences. Accordingly, Catto *et al.* demonstrated that methylation levels increase with advanced tumor stage, possibly leading to an easier detection of these tumors.[18] Dulaimi *et al.* reported a sensitivity of 87% for their gene panel (*APC*, *RASSF1A*, *p14 (ARF)*. Although they did not record whether primary or recurrent samples were used 28/45 tumors were classified as muscle-invasive.[27] In the same line, recently Renard *et al.* developed a two-gene panel (*TWIST1* and *NID2*) to identify tumors in voided urine samples of 466 primary bladder cancer patients (sensitivity 90%; specificity 93%). Notably, most studies use urine from patients without bladder cancer as a control group, but we believe that this does not reflect the true clinical practice where patients with a history of BC do not have the same risk probability of developing a recurrent tumor compared to control patients without a history of bladder cancer. The selection of our gene panel was based on recurrent NMIBC tumors and the test was validated on a subset of urines from BC patients with no recurrence at the time of urine collection.

Recurrent tumors (mainly TaG1-2, and one T1G2) were not detected in 32% of the cases (18/49+18/65) by the methylation assay. Possibly, tumor cells were absent at the time of urine collection or the number of urinary tumor cells was below the detection threshold of the assay. We show in a recent study that this might partly be solved by regular urine sampling (manuscript submitted). Secondly, we also demonstrated that not all tumors are methylated, hence stratification according to methylation status of the primary tumor could improve the sensitivity of the test (unpublished data).

"False" positive urines were detected in 33% (25/56+13/60) of cases (Table 4.4). Firstly, BC appears to arise from a field of change that affects not only macroscopically visible tumors, but also the area surrounding the tumor.[34] It might be possible that even though no tumor is detected cystoscopically, urothelial cells in the surrounding area of the resected tumor contain epigenetically changed cells. Secondly, multiple studies showed residual tumor in 35-81% at the same site of the initial resection at the first follow-up cystoscopy or at deep resection of the tumor base.[35-37] Thirdly, it is known that cystoscopy does not detect all tumors and recent studies show that the sensitivity of detection of NMIBC by the currently used white light cystoscopy varies between 68-83%. These studies also demonstrate that blue light fluorescence cystoscopy has superior sensitivity and studies on micro-satellite analysis demonstrate an increased sensitivity of cystoscopy when the urologist was aware of a positive test outcome.[5-7, 38]

Most biomarkers have a chosen cut-off point to distinguish positive (detected) from negative (missed) test results. However, dichotomizing markers before adding them to a statistical model generally leads to loss of data, resulting in reduced accuracy of the developed test. We designed a logistic regression model, based on raw methylation input data, to predict the probability of a recurrent tumor. A cut-off probability of 0.34 was selected for having the highest sensitivity and specificity.

Limitations of this study are the absence of tumor material for the test and validation subsets. Possibly, methylation was absent in the tumor, which might affect the sensitivity of the assay. Furthermore, our assay contains 23 probes, which might influence the efficiency of probe hybridization. Therefore, we selected a gene panel and our next aim is to develop a BC MS-MLPA, containing only the selected gene probes.

CONCLUSIONS

This is the first study to develop a methylation assay specific for the detection of NMIBC recurrent tumors in voided urine. These patients have small recurrences that require development of highly sensitive tests. Although at this time the sensitivity and specificity of the test are not sufficient for clinical implementation, our findings are promising and improvement of this test might eventually contribute to a more individualized patient-friendly surveillance. Additionally it would be of interest to perform functional studies to determine whether promoter methylation of the selected panel is related to gene silencing.

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Chapter 5

Combinations of urinary biomarkers for surveillance of patients with incident non-muscle invasive bladder cancer The European FP7 UROMOL Project

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ABSTRACT

Objective | To determine a combination of markers with optimal sensitivity for detection of recurrences in voided urine after resection of a primary low-grade non-muscle invasive bladder tumor (NMIBC).

Material and methods | Patients with NMIBC G1/G2 (n=136) were included at trans-urethral resection of the primary tumor. At least three follow-up urine samples were required for patient selection. DNA was extracted from the primary tumor and cell pellets of the subsequently collected urine samples. FGFR3, PIK3CA and RAS mutation analysis, micro-satellite analysis (MA) and methylation analysis (MS-MLPA) were performed on the tissue and urine DNA samples.

Results | We obtained 716 urine samples. The 136 patients experienced 552 recurrences during a median follow-up of three years. Sensitivity for detection of a recurrent tumor varied between 66% and 68% for the molecular tests after stratification of the patients based on tumor DNA analysis. A combination of markers increased sensitivity but reduced the number of patients eligible for a certain test combination. Combination of urine cytology with *FGFR3* analysis, without stratification for *FGFR3* status of the primary tumor, increased sensitivity from 56% to 76%.

Conclusions | A combination of markers increases the percentage of patients eligible for urine-based follow-up and increases the sensitivity of recurrence detection. Addition of *FGFR3* analysis to urine cytology could be valuable for the non-invasive follow-up of NMIBC patients.

INTRODUCTION

Cystoscopy is the gold standard for the follow-up of non-muscle invasive bladder cancer (NMIBC) patients and the cystoscopy frequency is determined by the recurrence and progression risk according to EAU guidelines.[1] However, cystoscopy is a time-consuming procedure that causes physical discomfort for patients and the sensitivity has been estimated to vary from 68-83%.[2, 3]

After resection, up to 70% of patients with NMIBC develop one or multiple recurrences. Hence, patients have frequent trans-urethral resections of the tumor (TUR) and together with the abundant cystoscopies this makes bladder cancer one of the most expensive tumor types.[4, 5] In addition to cystoscopy, urine cytology is performed, but its sensitivity for the detection of low-grade tumors is low.[6] Nevertheless, cytology is still used as a reference in the absence of highly sensitive urinary biomarkers. Up to now, multiple studies have been performed, usually investigating one type of urinary maker. Various markers proved to be more sensitive than cytology, however none of the single type of markers reached a sensitivity high enough to safely reduce the number of cystoscopies. A major problem with many studies that test biomarkers for urine diagnosis is that the investigated urine samples are often derived from patients with primary tumors, which are mostly larger than recurrent tumors and are also of higher grade. This results in high sensitivities and subsequently the marker sensitivity drops considerably when tested on urine samples derived from patients with a recurrent tumor.[7] Patients that are eligible for urine-based surveillance will typically present with NMIBC of G1 or G2. The risk of progression in these patients is low; however the risk of multiple recurrences is high.

Previous studies have demonstrated the role of hypermethylation, leading to transcriptional silencing of tumor suppressor genes and aberrant methylation has been detected in voided urine of patients with BC, which appears to be more sensitive than urine cytology.[8-13] Recently, we have developed a methylation assay, i.e. bladder cancer specific multiplex ligation-dependent probe amplification (MS-MLPA) assay. This methylation assay is specifically developed for the detection of NMIBC recurrences in voided urine.[14] The use of micro-satellite analysis (MA) for the detection of loss-of-heterozygosity (LOH) in voided urine samples is also reported in multiple studies.[15-17] Van der Aa *et al.* demonstrated that the positive predictive value of MA was higher in patients without mutations in the fibroblast growth factor receptor-3 (*FGFR3*).[15] Mutations in *FGFR3*, leading to constitutive activation of the RAS-MAPK pathway, have been found in 70% of NMI bladder tumors and are associated with overall favorable prognosis.[18, 19] *RAS* mutations (*KRAS, HRAS, NRAS)* have been found in 13% of bladder tumors of all stages and grades, being mutually exclusive with *FGFR3* mutations.[20] Previous research demonstrated *PIK3CA* mutations in 13-27% of the bladder tumors, whereas *PIK3CA* mutations mostly co-occurred with *FGFR3* mutations.[21] Recently, we showed that urinary *FGFR3* mutation analysis in patients with a primary *FGFR3* mutant (MT) tumor is a suitable assay for the detection of recurrences.[22] Additionally, we found that 88% of the low-grade NMIBC patients harbored a mutation in one of the mentioned oncogenes, making these patients eligible for urine-based surveillance by mutation detection assay.[23]

The *FGFR3, RAS, PIK3CA* and MS-MLPA assays are multiplex assays that can either detect a mutation or a methylated gene. Hence, they represent a single assay. It is not possible to multiplex the MA, making this assay more labor intensive. Further developments like next-generation sequencing would facilitate the combination of assays. Therefore, it is worthwhile to test whether a combination of assays would be better than a single.

The objective of this study was to determine a combination of markers with optimal sensitivity in order to detect recurrences in voided urine. Therefore, we used mutation analysis of *FGFR3, PIK3CA, HRAS, KRAS, NRAS,* in combination with methylation-specific MLPA and MA assays to increase the percentage of patients that could be monitored by urine analysis. The results were compared to urine cytology in a large retrospective longitudinal cohort. This study was part of the European FP7 UROMOL project.

MATERIAL AND METHODS

Patient material

A total of 716 voided urine samples from 136 patients with NMIBC (Ta/T1, G1/2)[24] were collected at the Department of Urology at Aarhus University Hospital at trans-urethral resection of the primary tumor. Patients with a history of carcinoma in situ were excluded from participation. 10-50 mL urine was collected at regular follow-up visits. Urine specimens were collected immediately before cystoscopy; cells were sedimented by centrifugation, and frozen at -80°C. Tumor biopsies were stored at -80°C immediately following resection of the tumor. A cystoscopic examination coincided with urine collection for molecular analysis. The availability of at least 3 follow-up urine samples, were a prerequisite for study inclusion. Recurrence was defined as a histologically proven tumor. Progression was defined as progression to muscle-invasive disease. Informed written consent was obtained from all patients, and research protocols were approved by the Central Denmark Region Committees on Biomedical Research Ethics.

DNA isolation and molecular analyses

DNA from tumor biopsies was extracted using Qiagen Dneasy blood and tissue kit (Qiagen, GmbH, Hilden Germany). DNA was extracted from urine cell pellets using the QiAamp Mini and Blood mini kit (Qiagen GmbH, Hilden, Germany).

Mutation analysis for *FGFR3, PIK3CA, RAS* was performed as described previously.[22, 23, 25] LOH was detected by micro-satellite analysis using primers for 12 polymorphic micro-satellite markers localised on chromosomes 8, 9, 10, 11 and 17 (van Tilborg *et al*., submitted). The MS-MLPA was used for the detection of methylation as described by Nygren *et al,*[26] This MS-MLPA was specifically developed for the detection of bladder cancer recurrences[14]. All details of primers, probes and conditions are given in Supplementary File 5.1.

Statistical analysis

The Predictive Analytics Software 17 (SPSS Inc, Chicago, Illinois, USA) was used for data analysis. Statistical differences were considered significant if $p < 0.05$. The Chi-square and Fisher's exact test were used to determine the relationships between different variables.

RESULTS

Patient and tumor characteristics

Patients with primary NMIBC G1 or G2 (n=136) were included for molecular analyses. Patients with G3 primary tumors were excluded because of their high risk of progression. Included patients had a total of 552 recurrences during a median follow-up of three years. Patient and primary tumor characteristics are depicted in Table 5.1. Four patients had progression to muscle-invasive disease. From 93/136 patients, there was DNA available from the primary tumor and from 22/136 patients, there was DNA available from the tumor at first visit. Tumors were analyzed for mutations in *FGFR3*, *PIK3CA*, *RAS (HRAS, KRAS, NRAS)* and the presence of LOH and methylation. *FGFR3* mutations were detected in 68% (78/115), *PIK3CA* 18% (20/111) and *RAS* 9% (10/110). *PIK3CA* and *FGFR3* mutations co-occurred in 16/20 (80%) cases, whereas *FGFR3* and *RAS* mutation were overall mutually exclusive, since only one patient harbored both mutations. Combined, 79% (91/115) of the patients had a mutation either in *FGFR3, PIK3CA* or *RAS* (Figure 5.1). LOH was detected in 63% (72/114) and methylation in 98% (88/90) of the patients. *FGFR3* mutations or LOH were detected in 81% of the primary tumors. During follow-up, 552 histologically proven recurrences were detected. These included mainly stage Ta (92%), G1/2 (82%) and solitary tumors (67%) (Table 5.2). Overall, recurrent tumors in patients with *FGFR3* mutant primary tumor were of lower stage (Figure 5.2a, p=0.001) and lower grade (Figure 5.2b, p=0.003) when compared to recurrent tumors from patients with an *FGFR3* wild-type primary tumor. No upper tract recurrences were detected.

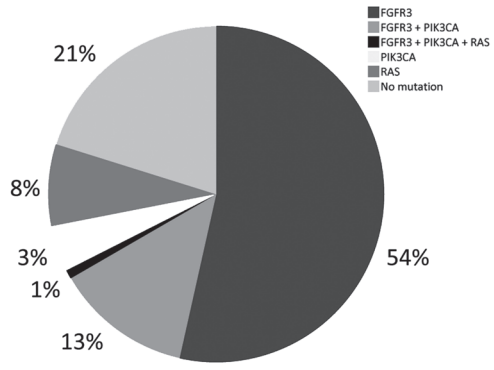

Figure 5.1 *Mutation status of 115 incident tumors. 79% harboured an activating mutation in at least one of the tested oncogenes and 21% had no mutation.*

Table 5.1: Clinical and molecular characteristics of 136 patients and primary tumors

	n (%)		n (%)
Gender		*FGFR3*	
Male	90 (66)	Wild-type	37 (32)
Female	46 (34)	Mutant	78 (68)
		PIK3CA	
Smoking		Wild-type	91 (82)
No	47 (35)	Mutant	20 (18)
Yes	70 (51)	*RAS*	
Unknown	19 (14)	Wild-type	100 (91)
		Mutant	10 (9)
Stage		*FGFR3* + *PIK3CA*	
Ta	120 (88)	Mutant	16 (14)
T1	16 (12)	*FGFR3* + *RAS*	
		Mutant	1 (0.9)
Grade		*FGFR3* + *PIK3CA* + *RAS*	
G1	34 (25)	Mutant	1 (0.9)
G2	102 (75)	*FGFR3, PIK3CA* or *RAS*	
		Wild-type	24 (21)
Multiplicity		Mutant	91 (79)
Solitary	104 (76)	LOH	
Multiple	32 (24)	No	42 (37)
		Yes	72 (63)
Tumor Size*		*FGFR3* or LOH	
<3 cm	97 (71)	No	22 (19)
>3 cm	38 (28)	Yes	93 (81)
Unknown	1(1)	Methylation	
		No	2 (2)
Progression		Yes	88 (98)
No	132 (97)	*FGFR3* or Methylation	
Yes	4 (3)	No	1 (1)
		Yes	88 (99)

** In case of multiplicity, tumor size was determined by largest tumor*
LOH = Loss of Heterozygosity

Table 5.2: Clinical characteristics of 552 recurrent tumors

	n (%)
Stage	
Ta	506 (92)
T1	30 (5)
T2-T4	9 (2)
Tx	7 (1)
Grade	
G0	17 (3)
G1	155 (28)
G2	298 (54)
G3	63 (11)
Gx	19 (3)
Multiplicity*	
Solitary	434 (79)
Multiple	118 (21)
Tumor size	
<3	523 (96)
>3	17 (3)
unknown	7 (1)

** In case of multiplicity, tumor size was determined by largest tumo*

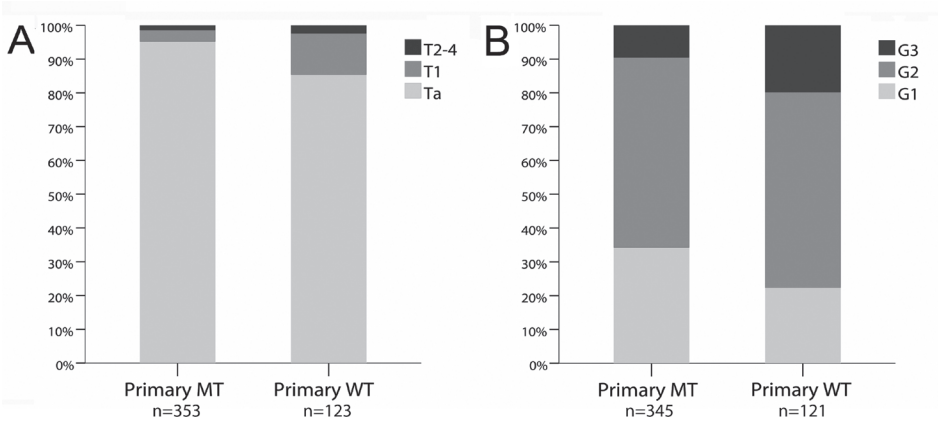

Figure 5.2 *Stage (A) and grade (B) of 482 recurrent tumors. Patients were stratified by FGFR3 mutation status of incident tumor. Sixpatients with Tx and 16 with Gx were not included in analysis. WT= wild-type, MT = mutant.*

Test performance of all single and combination of urine tests for the detection of recurrent tumors

Based on the molecular characteristics of the primary tumors, a combination of *FGFR3* mutation analysis with other mutation assays, MA or MS-MLPA increased the percentage of patients suitable for urine-based follow-up. 99% of all urine samples could be analyzed for *FGFR3* mutations. Due to the limited amount of DNA in some of the urine samples, only 70% could be analyzed for LOH and 63% for methylation. In 20% of all urine samples there was DNA left for the analysis of *RAS* and *PIK3CA* mutations. For each single and combination of assays the sensitivity for the

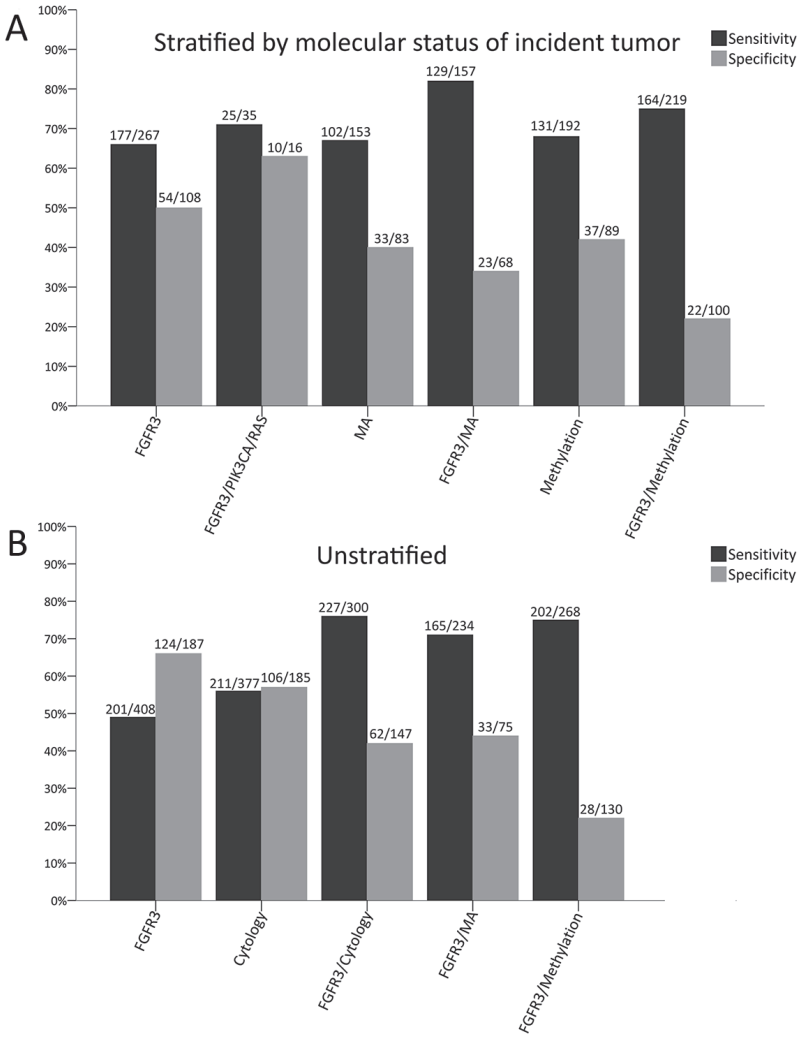

Figure 5.3 *Sensitivity and specificity of all single and combined tests with (A) and without (B) patient stratification based on the molecular status of the primary tumor*

detection of a recurrent tumor in voided urine was determined. Patients were stratified according to the molecular status of the primary tumor and analyses were done for each subgroup. Calculation of the sensitivity for *PIK3CA* or *RAS* mutation analysis in recurrent tumors separately was omitted because of the low numbers of urine samples available for analysis. For MA, we also calculated the sensitivity when using only the five markers for loss on chromosome 9. Sensitivity of all single and combined tests is shown in Figure 3a. The sensitivity for the detection of recurrences in patients with an *FGFR3* mutant primary tumor (stratified) was 66%. Adding *PIK3CA* and *RAS* assays to *FGFR3* mutation analysis increased the sensitivity for recurrence detection from 66% to 71%. However, only a limited amount of samples could be analyzed for PIK3CA and RAS mutations. The combination of *FGFR3*/MA had the highest sensitivity (82%) for the detection of recurrences. For this combination, patients were stratified based on an *FGFR3* mutation in their primary tumor and/or LOH in the primary tumor. This analysis was possible for 81% of the patients. The analysis with 12 different MA markers requires a high amount of DNA; therefore we also calculated the sensitivity for the detection of recurrent tumors using only the five markers for loss on chromosome 9. The sensitivity of MA was now 64% compared to 67% for analysis with all 12 markers. Combined with *FGFR3* analysis the sensitivity increased to 76%. For the combination of *FGFR3*/cytology patients were not stratified based on their primary tumor, this combination reached a sensitivity of 76%. Overall the specificity for all single and combined tests was low ranging from 34% (*FGFR3*/MA) to 66% (*FGFR3 unstratified).*

Molecular analyses are superior to urine cytology for the detection of low stage and low grade recurrences

The overall sensitivity of all single and combined molecular tests for the detection of recurrent tumors was higher than the sensitivity of urine cytology alone (Figure 5.3). Combining urine cytology with *FGFR3* analysis improved the sensitivity from 56% to 76%. Table 5.3 shows the

Table 5.3: Test sensitivity stratified and not stratified by molecular status of primary tumor

	Detected Stage/Total (% sensitivity)				Detected Grade/Total (% sensitivity)				
	Ta	T1	T2-4	Tx	G0	G1	G2	G3	Gx
Stratified:									
FGFR3	164/253 (65)	8/9 (89)	4/4 (100)	1/1 (100)	7/12 (58)	52/80 (65)	97/143 (68)	18/23 (78)	3/9 (33)
MA	92/139 (66)	4/6 (67)	6/7 (86)	-	3/5 (60)	31/47 (66)	56/82 (68)	11/16 (69)	1/2 (50)
Methylation	122/180 (68)	8/9 (89)	1/2 (50)	0/1 (0)	1/5 (20)	29/51 (57)	79/107 (74)	18/22 (82)	4/7 (57)
FGFR3/MA	137/163 (84)	2/2 (100)	3/4 (75)	-	6/6 (100)	46/54 (85)	79/91 (87)	6/12 (50)	5/6 (83)
FGFR3/methylation	153/204 (75)	6/9 (66)	3/4 (75)	2/2 (100)	8/9 (89)	53/70 (76)	79/110 (72)	19/24 (79)	5/6 (83)
Not stratified:									
FGFR3	186/381 (49)	10/19 (53)	4/5 (80)	1/3 (33)	8/16 (50)	60/117 (51)	109/219 (50)	21/42 (50)	3/14 (21)
Cytology	185/344 (54)	16/19 (84)	6/8 (75)	4/6 (67)	6/13 (46)	33/99 (33)	127/203 (63)	37/45 (82)	8/17 (47)
FGFR3/cytology	208/279 (75)	12/13 (92)	5/5 (100)	2/3 (67)	9/13 (69)	52/79 (66)	130/162 (80)	30/33 (91)	6/13 (46)
FGFR3/MA	155/195 (79)	5/6 (83)	3/4 (75)	2/2 (100)	7/7 (100)	50/63 (79)	91/113 (81)	10/16 (63)	7/8 (88)
FGFR3/methylation	189/249 (76)	7/12 (58)	4/5 (80)	2/2 (100)	9/10 (90)	66/86 (77)	98/133 (74)	24/33 (73)	5/6 (83)

sensitivities for all tests stratified according to stage and grade. The sensitivity for the detection of high stage and high grade tumors was similar in both the combined molecular tests and urine cytology. However in the case of low stage and low grade tumors, both single and combined molecular tests displayed higher sensitivity when compared to urine cytology. Adding *FGFR3* analysis to urine cytology improved the sensitivity for the detection of low stage and low grade tumors i.e. Ta 54% to 75%, G1 33% to 66%.

DISCUSSION

In this study, we investigated whether molecular analyses could play a role in the surveillance of patients presenting with G1-2 NMIBC. Activating point mutations in *FGFR3, PIK3CA* and *RAS,* LOH and methylation have been found in bladder tumors of all stages and grades. These genetic and epigenetic alterations could be used for early recurrence detection in voided urine, possibly reducing the number of cystoscopies, resulting in a less invasive follow-up. Molecular characterization of the primary tumor could be used to stratify patients for an appropriate follow-up schedule according to the recurrence and progression risk of that specific molecular profile. In this study, *FGFR3, PIK3CA, RAS* mutation detection, MA and MS-MLPA were evaluated for the detection of bladder cancer recurrences in voided urine.

The sensitivity of *FGFR3* analysis (66%) for concomitant recurrence detection in urine of patients with an *FGFR3* MT primary tumor was comparable to previous reports.[22] That not all recurrent tumors are detected is mainly the result of the number of tumor cells being below the analytical sensitivity of the assays. In a previous study we showed that sensitivity of the *FGFR3* assay for tumors larger than 1.5 cm was 100% and 75% for tumor smaller than 1.5 cm. Analyzing more than one urine sample could increase the sensitivity[27]. A combination of *FGFR3* analysis with other molecular tests improved the sensitivity. A total of 79% of the tumors were mutant for *FGFR3, PIK3CA and/*or *RAS*. Although, we could only analyze a limited number of samples for this combination; the combined sensitivity of the three mutation assays was 71%. The optimal combination for recurrence detection was *FGFR3*/MA based on stratification of the primary tumor for the presence of *FGFR3* mutations and/or LOH. Without stratification of patients based on molecular status of the primary tumor, the combination of *FGFR3*/cytology reached a sensitivity of 76%. Cytology is known to have a low sensitivity for G1 (7%-38%) and G2 (18%-46%) tumors.[28] Hence, the combination of cytology with the *FGFR3* mutation assay, which is more directed towards low grade/stage tumors, for surveillance is an attractive idea.

Overall the specificity of all single and combined tests was low. In principle, the specificity of *FGFR3* and other mutation assays is 100% since these mutations do not occur in sufficient amounts in non-bladder cancer controls.[29] The low specificity of molecular tests in cohorts under surveillance for recurrent bladder cancer could partly be explained by the so called anticipatory effect of the urine analysis, i.e. the urine test identifies a recurrence earlier than cystoscopy.[22] For example in 85% (46/54) of the patients in this study with a 'false positive' FGFR3 analysis,

a recurrent tumor was detected within 12 months. In addition, cystoscopy misses tumors since the currently used white light cystoscopy has been estimated to have a sensitivity between 68%-83%.[2, 3]

Clinical implementation of a molecular test is not only dependent on the performance of the marker, but also dependent on the costs, specifically since bladder cancer is an expensive cancer. Mutation analysis is cheap with consumables under $10 per sample, including DNA isolation, and the tests can be performed in a standard laboratory. Additionally, the tests require only 5ng of DNA. Based on our finding in this retrospective study, 99% of the urine samples would contain sufficient DNA to perform the *FGFR3, PIK3CA* and *RAS* mutation assays. Although MA by itself is cheap, the test requires 12 individual PCR reactions per sample and the analysis of the results is laborious and would need automation before being implemented in routine clinical practice. Moreover, a substantial percentage of urine samples will not yield sufficient DNA to perform MA. However the amount of markers could be reduced by using only the five markers for chromosome 9. The markers in the MS-MLPA kit have a high predictive value. Unfortunately, in our hands the test appeared not to be a suitable technology for introduction into clinical practice. The reason for this is that in many cases the PCR step failed and no product was apparent on the sequencer.

Strengths of the study include the analysis of a substantial number of primary tumors together with at least three follow-up urine samples per patient. Only a limited number of urine samples could be analyzed for *PIK3CA* and *RAS* mutations, because the study had already started when these assays were under construction. The findings presented here need to be validated in a large prospective study, which is currently being done in the European FP7 UROMOL project.

CONCLUSIONS

This is the first study to develop an optimal combination of urine-based tests in order to increase the sensitivity of recurrence detection. All tests had a higher sensitivity for low stage and grade disease compared to urine cytology. Therefore, a combination of molecular tests or a combination of the *FGFR3* assay with cytology represents promising possibilities for the follow-up of low grade NMIBC patients. These findings will be validated in a large prospective multicenter study of the European FP7 UROMOL project.

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SUPPLEMENTARY FILES

SNaPshot mutation analysis

Mutation analysis of *FGFR3, PIK3CA* and *RAS* was performed as described by van Oers et al[1]. A multiplex PCR was performed in a volume of 15µl, containing 1x PCR buffer, 1.5mM $MgCl_2$, 0.5 units Taq DNA polymerase (Promega, Madison, WI), 0.17mM deoxynucleotide triphosphates (Roche, Basel, Switzerland) 5% Glycerol (Fluka, Buchs SG, Switzerland) and 5ng of DNA and 1 µl of PCR primer mix (final concentrations of primers are depicted in supplementary table 5.1). The PCR conditions were as follows: 5 minutes at 95°C followed by 35 cycles at 95°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. The PCR was completed with 10 minutes at 72°C. Next products were treated with 3 units of shrimp alkaline phosphatase and 2 units of Exonuclease I (SAP and ExoI, Amserham Biosciences, Uppsala, Sweden). After the removing of the excess primers and deoxynucleotide triphosphates, PCR-products were analyzed for mutations by using the ABI PRISM SnaPshot Multiplex kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The SNaPshot method is based on dideoxy single-base extension of unlabeled oligonucleotide primers. For each mutation a primer annealing adjacent to the potentially mutant nucleotide was developed. The different in length of poly(dT)-tail attached to the 5'-end enabled the simultaneous detection of the different primers. The multiplex SNaPshot reaction was performed in a volume of 10µl, containing 1µl PCR product, 2.5µl Ready Reaction mix, 1x sequencing buffer, and 1µl SNaPshot Primer mix (final primer concentrations are depicted in supplementary table 5.2). The cycling scheme consisted of 35 cycles of 10 seconds at 96 °C and 40 seconds at 58.5°C. In the final step 1µl shrimp alkaline phosphatase was added. Products were analyzed using an automatic sequencer (ABI 3130, Genetic Analyzer, Applied Biosystems).

Microsatellite analysis

Microsatellite analysis (MA) was performed as described by van Tilborg et al (submitted, 2012). Shortly, PCR of 12 different microsatellite markers was performed with separate primer pairs of which 1 oligonucleotide was labelled at the 5-end with fluorescent dyes 6-FAM (Invitrogen, the Netherlands). Primer sequences are depicted in supplementary table 5.3. PCR was carried out in a total volume of 15µl, consisting of 5x GoTaq PCR buffer, 2.5 mM $MgCl_2$ (Promega) 0.2 mM dNTP's (Boehringer), and 5 units GoTaq DNA polymerase. For each marker a DNA input of 5ng was required. The following cycling conditions were used: 95°C for 5 minutes followed by 28 cycles at 95°C for 45 seconds, 55°C at 45 seconds and 72°C for 45 seconds and the final step 10 minutes at 72°C. Next DNA fragments were analyzed using an automatic sequencer (ABI 3130, Genetic Analyzer, Applied Biosystems). All samples were analyzed in duplicate. We applied a lower limit for peak heights of 700 to exclude possible preferential amplification of one of the two alleles due to insufficient input DNA. All reactions with off-scale signals were diluted and rerun. A result was only taken into account when at least four MA markers in a sample were informative.

Based on findings from a previous study (Tilborg et al, submitted) cut-off values for each marker were set to 95% specificity based on urine samples from healthy individuals. The upper and lower cut-off values are depicted in supplementary table 5.4. The mean peak ratio per sample was calculated.

Bladder Cancer Specific MS-MLPA

DNA amplicons were prepared for hybridization according to MS-MLPA protocol, originally described by Nygren et al[2]. MLPA reagents were obtained from MRC-Holland, Amsterdam, the Netherlands (KIT-ME014). Probe sequences are depicted in supplementary table 5.5. Briefly, 50-150ng of genomic DNA was denatured for 10 minutes at 98°C. 1.5µl SALSA MLPA-buffer and 1.5µl probe mix were added and the mixture was incubated for 1 minute at 95°C, followed by a 16-hours incubation at 60°C. This incubation allowed the probes to bind to their respective targets. After hybridization a mix of 10µl water and 3µl Ligase-buffer A was added to each sample, resulting in a total volume of 20µl. 10µl of this volume was then transferred to an adjacent well to ensure duplicate analysis. The 96-wells plate was incubated for 1 minute at 49°C followed by the addition of Ligase-65 Mix. This mix contained 1.5µl Ligase-65 buffer B, 8.25µl water, 0.25µl Ligase enzyme and 0.5µl HhaI enzyme (Promega R6441, 10 units/µl). Simultaneous ligation and digestion was then performed by incubation for 30 minutes at 49°C, followed by a 98°C ligase inactivation treatment of 5 minutes. Finally 5µl of ligation product was added to a PCR mixture, consisting of 1µl SALSA PCR-primers, 1µl SALSA Enzyme Dilution buffer, 2µl SALSA PCR buffer, 15.75µl water, and 0.25µl SALSA polymerase. Ligation products were amplified by PCR for 35 cycles: 30 seconds 95°C; 30 seconds 60°C; 60 seconds 72°C. The PCR was completed with 20 minutes incubation at 72°C. Products were analyzed using an automatic sequencer (ABI 3130, Genetic Analyzer, Applied Biosystems).

A combination of probes was selected using the least absolute shrinkage and selection operator (LASSO) method. Probes with non-zero coefficients in the LASSO were combined into a gene panel. This gene panel was used in a logistic regression model to determine the probability for having a recurrence based on methylation of this selected gene panel: (APC_a * 0.097) + (TERT_a * 0.507) + (TERT_b * 0.218) + (EDNRB * 0.501) – 2.480. A probability threshold of 0.34 was selected to indicate whether a test outcome was considered positive or negative[3].

Supplementary table 5.1: Primer sequences mutation analysis

FGFR3 PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
FGFR3 RI Fw	AGTGGCGGTGGTGGTGAGGGAG	115	1.2
FGFR3 RI Rev	GCACCGCCGTCTGGTTGG		1.2
FGFR3 RII Fw	CAACGCCCATCTCTTTGCAG	138	0.7
FGFR3 RII Rev	AGGCGGCAGAGCGTCACAG		0.7
FGFR3 RIII Fw	GACCGAGGACAACGTGATG	160	0.7
FGFR3 RIII Rev	GTGTGGGAAGGCGGTGTTG		0.7
Pan-RAS PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
HRAS exon1 Fw	CAGGAGACCCTGTAGGAGG	139	0.6
HRAS exon1 Rev	TCGTCCACAAAATGGTTCTG		0.6
HRAS exon2 Fw	GGAGACGTGCCTGTTGGA	140	0.3
HRAS exon2 Rev	GGTGGATGTCCTCAAAAGAC		0.3
KRAS exon1 Fw	GGCCTGCTGAAAATGACTG	163	0.3
KRAS exon1 Rev	GGTCCTGCACCAGTAATATG		0.3
KRAS exon2 Fw	CCAGACTGTGTTTCTCCCTT	155	0.3
KRAS exon2 Rev	CACAAAGAAAGCCCTCCCCA		0.3
NRAS exon1 Fw	GGTGTGAAATGACTGAGTAC	128	0.3
NRAS exon1 Rev	GGGCCTCACCTCTATGGTG		0.3
NRAS exon2 Fw	GGTGAAACCTGTTTGTTGGA	103	0.3
NRAS exon2 Rev	ATACACAGAGGAAGCCTTCG		0.3
PIK3CA/NRAS PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
PIK3CA ex9-Fw	AGTAACAGACTAGCTAGAGA	139	1
PIK3CA ex9-Rev	ATTTTAGCACTTACCTGTGAC		1
PIK3CA ex20-Fw	GACCCTAGCCTTAGATAAAAC	109	0.7
PIK3CA ex20-Rev	GTGGAAGATCCAATCCATTT		0.7
NRAS ex.2 Fw	GGTGTGAAATGACTGAGTAC	128	0.3
NRAS ex.2 Rev	GGGCCTCACCTCTATGGTG		0.3
NRAS ex.3 Fw	GGTGAAACCTGTTTGTTGGA	103	0.5
NRAS ex.3 Rev	ATACACAGAGGAAGCCTTCG		0.5

Supplementary Table 5.2: Probe sequences mutation analysis

FGFR3 Probe mix

Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
S373C	T19 GAGGATGCCTGCATACACAC	39	sense	T	A	0.4
K652M/T	T20 CACAACCTCGACTACTACAAGA	42	sense	A	T/C	0.7
G372C	T29 GGTGGAGGCTGACGAGGCG	48	sense	G	T	0.2
A393E	T34 CCTGTTCATCCTGGTGGTGG	54	sense	C	A	0.7
R248C	T46 CGTCATCTGCCCCCACAGAG	66	sense	C	T	0.4
Y375C	T43 ACGAGGCGGGCAGTGTGT	61	sense	A	G	0.6
S249C	T36TCTGCCCCCACAGAGCGCT	55	sense	C	G	0.8
K652Q/E	T50 GCACAACCTCGACTACTACAAG	72	antisense	A	C/G	0.3
G382R	T56 GAACAGGAAGAAGCCCACACC	76	antisense	C	T	0.4

Ras Set 1 Probe mix

Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
HRAS pos.34	T17 CTGGTGGTGGTGGGCGCC	35	sense	G	C/T/A	0.5
HRAS pos.182	T18 GCATGGCGCTGTACTCCTCC	38	antisense	T	G/C/A	0.15
KRAS pos.34	T25 GGCACTCTTGCCTACGCCAC	45	antisense	C	G/A/T	0.5
HRAS pos.35	T31 CGCACTCTTGCCCACACCG	50	antisense	C	G/A/T	0.7
NRAS pos.182	T33 GACATACTGGATACAGCTGGAC	55	sense	A	G/C/T	0.5
KRAS pos.181	T41 CTCATTGCACTGTACTCCTCTT	63	antisense	G	T/C	0.2
HRAS pos.181	T46 CATCCTGGATACCGCCGGC	65	sense	C	A/G	0.7
KRAS pos.35	T49 AACTTGTGGTAGTTGGAGCTG	70	sense	G	C/T/A	0.2
HRAS pos 37	T55 CAGCGCACTCTTGCCCACAC	75	antisense	C	G/A/T	0.7
NRAS pos.34	T62 CTGGTGGTGGTTGGAGCA	80	sense	G	C/T/A	0.2

PIK3CA/NRAS Probe mix

Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
E542K	T17 ACACGAGATCCTCTCTCT	35	sense	G	A	0.15
E545G	T21 CCTCTCTCTGAAATCACTG	40	sense	A	G	0.5
E545K	T25 ATCCTCTCTCTGAAATCACT	45	sense	G	A	0.3
H1047R	T30 GAAACAAATGAATGATGCAC	50	sense	A	G	0.3
NRAS pos.34	T34 GTGCGCTTTTCCCAACACCAC	55	antisense	C	G/A/T	0.5
NRAS pos.35	T41 CTGGTGGTGGTTGGAGCAG	60	sense	G	C/A/T	0.3
NRAS pos.37	T46 GGTGGTGGTTGGAGCAGGT	65	sense	G	C/A/T	0.1
NRAS pos.38	T49 GTCAGTGCGCTTTTCCCAACA	70	antisense	C	G/A/T	0.5
NRAS pos.180	T54 GGACATACTGGATACAGCTGG	75	sense	A	T	0.3
NRAS pos.181	T58 CTCATGGCACTGTACTCTTCTT	80	antisense	G	C/T	0.2
NRAS pos.182	T63 GACATACTGGATACAGCTGGAC	85	sense	A	C/G/T	0.2
NRAS pos.183	T68 CTCTCATGGCACTGTACTCTTC	90	antisense	T	A/G/C	0.7

Supplementary table 5.3: primer sequences microsatellite markers

Marker	Size	Locus	Het	F/R	Sequence
D8S1109	143-167	8p	0.88	F	TCAGAATTGCTCATAGTGCAAGA
				R	ACTGTCTTGGTACATTTGTTTACCC
D8S1125	221-233	8p	0.69	F	CCCCCTAAAATTTAGCTCCA
				R	TATGCCTAGCCCTCCTTTCT
D8S1130	128-148	8p	0.94	F	GAAGATTTGGCTCTGTTGGA
				R	TGTCTTACTGCTATAGCTYYCATAA
D9S252	152-176	9q	0.75	F	CAAATTTGGCCTTGAACCAT
				R	AGCCCAGATATCCCCAAGTT
D9S299	178-198	9q	0.70	F	AAGTGTTGCATCAGAGCCTC
				R	AGTGTGAACATTATTTCATTCTGG
D9S304	135-175	9q	0.86	F	GTGCACCTCTACACCCAGAC
				R	TGTGCCCACACACATCTATC
D9S752	178-201	9q	0.73	F	CAGAGGTTGCAGTGAGCTA
				R	GCAAAGTCAGGCCATTATAC
D9S1118	141-177	9q	0.79	F	CAGGATATTATGTGATGGAATCC
				R	CTGCTGACTCCAAAAATATGC
D11S1981	134-178	11p	0.85	F	AATTCCTTTACTCCAGAAAGG
				R	CAGATTTCTGCTTTCCCAGA
D11S1999	109-137	11p	0.78	F	TACATGGCAGCAGGCATATA
				R	GAGATAAACAAGATTGCTAGATAGGC
D17S969	111-132	17q	0.73	F	ATCTAATCTGTCATTCATCTATTCA
				R	AACTGCAGTGCTGCATCATA
G10693	174-194	17p	0.94	F	ACATACAGCACAGGCCAAAT
				R	CCAGTCTTCCGTCACTATGC

* Concentration in reaction

Supplementary table 5.4: Lower en upper cut-off values microsatellite markers

Marker	95% interval
D8S1109	0.93-1.29
D8S1125	0.85-1.19
D8S1130	1.00-1.40
D9S252	0.97-1.23
D9S299	0.95-1.27
D9S304	0.86-1.33
D9S752	0.92-1.31
D9S1118	0.97-1.42
D11S1981	0.93-1.39
D11S1999	0.93-1.45
D17S969	0.95-1.33
G10693	1.00-1.29

Supplementary table 5.5: Probe sequences MS-MLPA kit ME014

124	TRAF4	TGCCAAGGTGAGTCCACACTGCCAGGA-AGAAGCCCAAGCACAGTCTGTTGCCCTTGGA
130	BCL2	TGTGAGATGTCCAGCCAGCTGCACCTG-ACGCCCTTCACCGCGCGGGGACGCTTTGCCA
136	PTEN	GCTCTTTCATTTTTAGGGCAAACGAGCCGAGT-TACCGGGGAAGCGAGAGGTGGGGCGCTGCAAG
142	TIMP3	TCCAGCGCCGAGGCAGCCTCGC-TGCGCCCCATCCCGTCCCGCCGGGCACTCGG
148	APC	GGCTGGGTGTGGGCGCACGT-GACCGACATGTGGCTGTATTGGTGCAGCCCGCCAGGGT
154	TERT	CGCAGCGCGGGGACCCGGCGGCTT-TCCGCGCGCTGGTGGCCCAGTGCCTGGT
160	TERT	TCTGTGTCCTGCCTGAAGGAGCTGGT-GGCCCGAGTGCTGCAGAGGCTGTGCGAGCGCG
172	WIF1	TCTGTCTAAACGGGAACAGCCCTGGCT-GAGGGAGCTGCAGCGCAGCAGAGTATCTGACGG CGCC
178	CCM2	CTGGAATTGTCTCGCCATTTAAACGAGTAT-TCCTAAAAGGTGAAAAGAGTAGAGATAAGAAAG CCCATGAGAAGGT
193	RARB	CCGCCGGCTTGTGCGCTCGCT-GCCTGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCT
202	MLH3	GCGACCTTGTTCTTCCTTTCCTTCCGA-GAGCTCGAGCAGAGAGGACTGTGATGAGACAGGA TAACAG
211	TGFBR1	GGCGTTACAGTGTTTCTGCCACCTCT-GTACAAAAGACAATTTTACTTGTGTGACAGATGGGCT CTGCTTT
220	COL2A1	GCCTCTTCCTTCTACACAGGGTCCTT-CTGGAGACCAAGGTGCTTCTGGTCCTGCTGGTCCTTCT
229	PAH	CAGTGCCCTGGTTCCCAAGAA-CCATTCAAGAGCTGGACAGATTTGCCAATCAGATTCTCAG
238	CDKN2A	CTGGATCGGCCTCCGACCGTAAC-TATTCGGTGCGTTGGGCAGCGCCCCCGCCTCCAGCAGC
247	PTCH	GTGGACAGCTGGGAGGAAATGCTGAA-TAAGGCTGAGGTTGGTCATGGTTACATGGACCGC CCCT
259	CDKN2A	CCAGGGGCACCAGAGGCAGTAACCATG-CCCGCATAGATGCCGCGGAAGGTCCCTCAGA
265	SLC2A1	CTCTGGTCCCTCTCAGTGGCCATCTTTTCT-GTTGGGGGCATGATTGGCTCCTTCTCTGTGGGCC TTTTCGTTAACC
283	BCL2	GTGAAGCGGTCCCGTGGATAGAGA-TTCATGCCTGTGCCCGCGCGTGTGTGCGCGCGTG
292	PTEN	CACCGGAGCGGGCGCAGGAGA-GGCCTGCGGGGTGCGTCCCACTCACAGGGAT
301	ESR1	GCTCGCGTGTCGGCGGGACAT-GCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCC
310	DBC1	CTGGAGGTTTGTTGAGCTCCTCTACT-TCCTGTTTATATGGGGCCGTATCTCAGTGCAGCCC
319	TIMP3	CATCGTGCTCCTGGGCAGCTGGA-GCCTGGGGGACTGGGGCGCCGAGGCGTGCACA
328	KRIT1	TGGTCTAGGAGCTCCAGACTACTCAAAAAT-CCAAATACCTAAACAGGAAAAATGGCAGAGAA GCATGAGCAGT
337	CDH1	CTATGAAGGAAGCGGTTCCGAAGCTGCTA-GTCTGAGCTCCCTGAACTCCTCAGAGTCAGAC AAAGACCAGGAC
346	TNFRSF25	CTGAAGGCGGAACCACGACGGGCA-GAGAGCACGGAGCCGGGAAGCCCCTGGGCGCCCG
355	TGFBR1	GAGAATGTTGGTATGCCAATGGAGCA-GCTAGGCTTACAGCATTGCGGATTAAGAAAACATTA TCGCA
364	DBC1	TCACTTCCTGACCATCGGAAGCATCCA-TCAGCACTGGGGCAATGACTGGGACCTGCAGA
375	ESR1	CCAGCCCGCCGTGTACAACTACCCCG-AGGGCGCCGCCTACGAGTTCAACGCCGCGGC
382	PTCH	GATAAGAGCTCCGGGGGGATTCTTCA-TGCACCAGTGTTTAGGGTACGTGCTTCCTAAGTAAAT CCAAACATTG
391	CDKN2A	GCTCTTCCGCCAGCACCGGAGGAA-GAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTG
400	TIMP3	CCAGCGCTATATCACTCGGCCGCCCA-GGCAGCGGCGCAGAGCGGGCAGCAGGCAG

Supplementary table 5.5: Probe sequences MS-MLPA kit ME014 (continued)

409	EDNRB	CCAAGTTTCCCACTGGCGCGCAA-ACTTGAGTTACTTTTGAGCGTGGATACTGGCGAAGAGG CTG
418	TERT	CCAGAGTTTCAGGCAGCGCTGCGTCCT-GCTGCGCACGTGGGAAGCCCTGGCCCCGGCC
427	APAF1	CCATTCATGCTTGTTTTGTTTTGGATTTTAGTAAGGACA-GTCCTGTGTGAAGGTGGAGTACCACA GAGGCCAGTTGTTTTTG
436	CDH13	GTTCTGTGCGTTCTCCTGTCCCAG-GTAGGGAAGAGGGGCTGCCGGGCGCGCTCTG
445	DBC1	GCAGTCACTCCTCTACTGTAATGAGAATGGGTT-TTGGGGAACCTTCCTGGAGAGCCAGCGGA GCTGCGTG
454	APC	CTCAGCTGTGTAATCCGCTGGATGCGGACC-AGGGCGCTCCCCATTCCCGTCGGGAGCCCGC
463	WIF1	CCTGCAAATAGAGCGAGAACAGAAGAGCGGGA-AGGGCTGGCGCGAGCGAGGTGCGAGCGAGGAG
472	CDH1	CTGAGGAGCGGAGCGGCCTGGAA-GCCTCGCGCGCTCCGGACCCCCCAGTGATGGGAGT
481	RARB	CCACATGTGCTTTTTCTGGAGTGGAAAAATACATA-AGTTATAAGGAATTTAACAGACAGAAAGG CGCACAGAGGAATTT

The Hha1 sites are marked in grey.

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Chapter 6

Validation of urinary biomarkers in a large multicenter patient cohort with low-risk bladder cancer: The European FP7 UROMOL project

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In preparation

ABSTRACT

Objective | Non-muscle invasive bladder cancer (NMIBC) accounts for 75-85% of all bladder cancer (BC). Recurrence develop in up to 70% and hence patients are followed with cystoscopy which has a sensitivity around 70%. A sensitive urine test would allow a reduction of these invasive endoscopic examinations. The aim of this study is to investigate the effect of combination of three urine assays for the detection of recurrent disease during the follow-up of patients with primary NMIBC.

Material and methods | This large prospective study comprised 1109 patients from four large European hospitals. Patients presenting with an initial tumor or in follow-up for BC were included from 2007 to 2012. Urine samples (n=2711) were collected prior to cystoscopy during regular follow-up visits. Marker performance was calculated for detection of concomitant recurrence. Anticipatory effect of urine analysis was visualized by Kaplan-Meijer curves.

Results | The combination of *FGFR3*, *TERT* and *OTX* urine analysis detected 57% of the recurrent tumors from patients with primary low-grade NMIBC. Sensitivity was 83% for recurrences that were grade 3 and/or ≥pT1 in this group. Sensitivity for recurrence detection in patients with primary high-grade disease was 72%. In addition, 76% of the positive urine samples were followed by a recurrence within 5 years. Most high stage recurrences were identified within 1 year after a positive urine sample. A limitation was the relatively small amount of urine samples that could be analyzed for *TERT* analysis.

Conclusions | The combination of assays increased recurrence detection in primary NMIBC patients and most aggressive tumors were identified. Furthermore, we observed that a positive urine assay was frequently followed by a recurrence. This study supports the value of urine analysis as an alternative for cystoscopies in patients with LG-NMIBC and as a valuable addition to the surveillance of patients presenting with HG tumors.

INTRODUCTION

Around 75%-85% of all patients presenting with bladder cancer will have non-muscle invasive disease (NMIBC).[1] Although the overall prognosis of NMIBC is favorable, these tumors tend to recur, with a 5-years recurrence rate ranging from 31-78%. Furthermore, there is a 1-17% chance of progression to muscle invasive disease, which is associated with a worse prognosis.[1] Since the high recurrence rate and the risk of disease progression, patients with NMIBC will be followed extensively by cystoscopy as defined by the guidelines of the European Association of Urology.[2] Cystoscopy is the gold standard for the detection of recurrent disease. Yet, the detection rate of white light cystoscopy is not optimal, with an estimated sensitivity varying between 46% and 80%.[3] Moreover, the procedure is expensive and invasive and causes discomfort.[4] In addition to cystoscopy, cytology is still frequently used, even though the additional value for the detection of low-grade tumors is poor.[5, 6]

Many studies have been performed searching for sensitive urine markers to detect NMIBC. Currently, none of the newly investigated markers are thought to be sufficiently sensitive to adjust the follow-up protocol. In addition, in many studies markers were tested on a mixture of urine samples derived from patients with an initial tumor and patients presenting with recurrent disease. Primary tumors are mostly larger in size and less differentiated than the recurrent tumor, causing more spilling of tumor cells, resulting in high marker sensitivity. When testing the marker in patients under surveillance sensitivity drop significantly.[7] Likewise, high-grade tumors are more likely to shed tumor cells compared to low-grade tumors and therefore the sensitivity will be much higher when investigating both low grade and high-grade tumors together compared to low-grade tumors alone.

We previously showed that the sensitivity of recurrence detection can be improved by combining several urine assays.[8] In this study we investigated three urine assays that showed their ability of recurrence detection in previous studies i.e. the *FGFR3* mutation assay, the *TERT* mutation assay, and the *OTX1* methylation assay. *FGFR3* mutations are present in approximately 70% of all NMIBC. Since these mutations are tumor specific, *FGFR3* mutation analysis proved to be an excellent marker for urine follow-up in patients with a primary *FGFR3* mutant tumor.[8-10] We recently showed that 70-80% of BCs harboured a *TERT* mutation and *TERT* mutation analysis in urine reached a sensitivity of 42% for recurrence detection.[11] The combination of *FGFR3* and *TERT* urine analysis improved the sensitivity to 50%. A methylation assay was recently developed by Kandimalla *et al* and contained probes for CpG-islands of three different genes, i.e. *ONECUT2*, *OTX1* and *OSR1*. This urinary assay reached a sensitivity of 74% for recurrence detection. *OTX1* was the best performing marker with a sensitivity of 65%.[12]

All three assays were studied in small patients series. This is the first study on urinary biomarkers involving a large prospective European patient cohort. The objective was to improve recurrence detection by combining the three promising assays, with the main focus on patients in follow-up for primary low-grade NMIBC. Furthermore, we aimed to elucidate the longitudinal effect of urine analysis.

MATERIAL AND METHODS

Patient and tissue collection

Between January 2007 and July 2012, patients treated for primary or recurrent bladder cancer were included from four different European hospitals i.e. Aarhus University Hospital, Aarhus, Denmark, Academic Hospital, Uppsala, Sweden, Centro Nacional de Investigaciones Oncológicas (CNIO) Madrid, Spain, and Erasmus Medical Centre, Rotterdam, The Netherlands. Patients from the Netherlands were checked in the opting-out system and tumor tissue and urine samples were used according according to the code of secondary use of human tissue (www.federa.nl). Informed written consent was obtained from all patients of the other hospitals. Research protocols were approved by the institutional review boards or ethical committees in all involved countries.

Urine samples were collected prior to cystoscopy during regular follow-up visits. Cells were sedimented by centrifugation within 12 hours after collection and frozen at -80ºC until further processing. Tumor biopsies were collected in TissueTEK O.C.T and immediately frozen at -80ºC. All tumors were histologically proven. A tumor found within 14 days after urine collection was defined as a concomitant tumor. Urine samples taken within 35 days after the previous urine sample were labeled as double and censured for data analysis. We distinguished a group of low-grade NMIBC (LG-NMIBC) that included patients with primary grade 1, grade 2, PUNLMP and low-grade BC and a group of high-grade NMIBC (HG-NMIBC) including patients with primary grade 3 and high-grade disease.

DNA Isolation and Molecular analyses

DNA from urine cell pellets was extracted by using the QIAamp mini and Blood kit (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol and tumor DNA was extracted by using the DNeasy® Blood and Tissue Kit. The *FGFR3* mutation analysis, *TERT* mutation analysis and methylation analysis were performed as described previously.[9, 12-15]. More details about experiment conditions, primer and probe sequences are given in the supplementary data.

Although we initially used five methylation markers we soon decided that this was unrealistic given the low amount of DNA available in many urine samples. Considering the results of Kandimalla *et al,* we decided to use only the marker for *OTX1*.[12] Based on our recent finding of *TERT* mutations in BC, we then assayed the samples with sufficient DNA left for the *TERT* mutation.[11] 90% of the urine samples yielded more than 25ng DNA, which is required to perform all three assays.

Data analysis

GeneMarker Software version 1.7 (SoftGenetics, State College) was used for the analysis of the SnaPshot data. Methylation percentage was calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peak multiplied by 100. Only methylation of *OTX1* was used for further data analysis. The determined cut-off value of 13.44 for the methylation percentage of *OTX1* allowed 20% false positives in the recurrent NMIBC

patients (80% specificity). *TERT* and *FGFR3* analysis were considered positive in case a mutant peak was present. For analysis of the combined marker sensitivity, a urine sample was marked positive when all assays were performed and at least one of the tested markers was positive.

T-test was used to calculate the difference in means and Chi-square test was used to compare sample distribution. KM-curves were used the present the anticipatory effect of the urine analysis. All statistical analyses were performed using the Statistical Package for Social Sciences version 22 (SPSS, Chicago).

RESULTS

Patients and urine samples

Between January 2007 and July 2012 a total of 2711 urine samples were collected from 1109 patients, comprising 858 (77%) males and 251 (23%) females. Patient and tumor characteristics are depicted in table 6.1. Sixty-five percent (720/1109) initially presented with LG-NMIBC. As

Table 6.1: Clinical and tumor characteristics of 977 patients included with primary NMIBC.

		Denmark (n=519)	Netherlands (n=231)	Sweden (n=103)	Spain (n=124)	Total (n=977)
Median Age at primary tumor (yrs)		65	65	69	70	66
Median FUP (yrs)		6,3	5,1	5,2	0,0	5,1
Gender	*Male*	393 (74%)	181 (78%)	78 (76%)	114 (92%)	766 (78%)
	Female	126 (26%)	50 (22%)	25 (24%)	10 (8%)	211 (22%)
Stage primary tumor	*Ta*	394 (76%)	175 (76%)	67 (65%)	91 (73%)	727 (74%)
	T1	125 (24%)	51 (22%)	34 (33%)	28 (23%)	238 (24%)
	Tis	-	5 (2%)	2 (2%)	5 (4%)	12 (1%)
	Missing	-	-		-	
Grade primary tumor	*G0*	8 (2%)	-	-		8 (1%)
	G1	82 (16%)	69 (30%)	26 (25%)	50 (40%)	227 (23%)
	G2	282 (54%)	119 (52%)	47 (44%)	28 (23%)	476 (49%)
	G3	139 (27%)	38 (16%)	25 (24%)	40 (32%)	242 (25%)
	G4	2	-	1	-	3
	PUNLMP	-	1	-	-	1
	LG	5 (1%)	-	1	1 (1%)	7 (1%)
	HG	-	-	1	-	1
	CIS	-	5 (2%)	2 (2%)	5 (4%)	12 (1%)
# concomitant recurrences	*LG- NMIBC*	251	101	81	18	451
	HG-NMIBC	79	17	24	7	127
# urines	*FUP - LGNMIBC*	1029	477	182	18	1706
	Primary - LGNMIBC	71	64	28	61	224
	FUP - HGNMIBC	352	56	70	7	485
	Primary - HGNMIBC	20	16	7	38	81

shown in figure 6.1, we collected a total of 1930 urine samples from these patients. 23% (257/1109) of the patients were included with primary HG-NMIBC, with a total of 566 urine

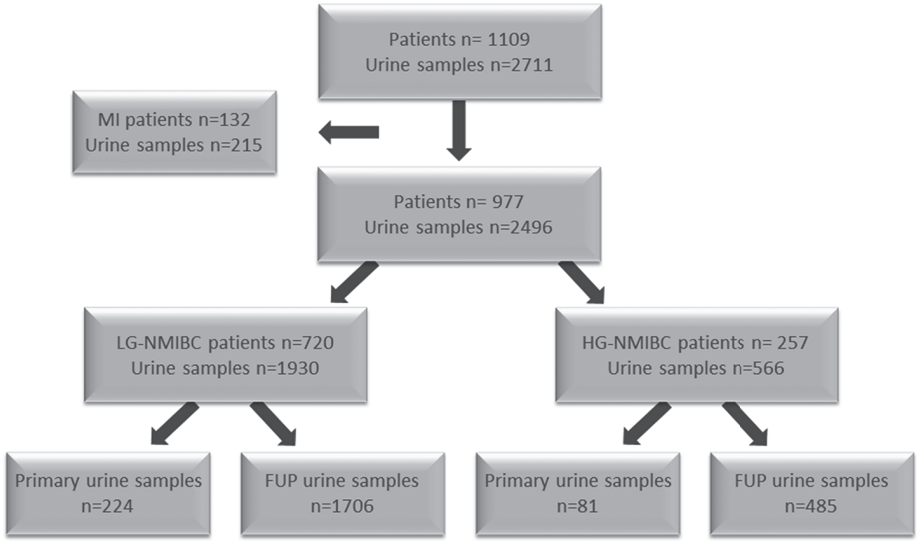

Figure 6.1 *Number of included patients and corresponding urine samples*

samples collected. Cytology data was available in 42% (1144/2713).

The diagnostic value of a combination of urine markers in the follow-up of NMIBC patients

HTERT mutation analysis reached highest sensitivity of 45% (147/324) for recurrence detection in patients with primary low-grade NMIBC, compared to 27% (107/403) for *FGFR3* mutation analysis and 38% (143/373/365) for *OTX1* methylation analysis. Urine cytology detected only 18% (21/114) of all recurrent tumors. Specificity for all three individual tests was relatively high: *FGFR3* 91% (1011/1113), *OTX1* 81% (800/993) and *HTERT* 70% (543/777). Combination of the three assays increased recurrence detection to 57% (163/285) with a specificity of 59% (392/668). All numbers are shown in Figure 6.2a. Most tumors missed by the combination of three assays were low stage and low grade i.e. 45% of the Ta tumors and 49% and 43% of grade 1 and 2 tumors (Table 6.2). On the other hand, most aggressive tumors were detected by urine analysis with the three-assay combination, i.e. 88% of the T1, 77% of the T2-4 tumors and 83% of the grade 3 tumors.

Analysis of urine samples collected during follow-up visits from patients with primary high-grade NMIBC (HG-NMIBC) revealed a higher rate of tumor detection for all markers and combination of markers, with the exception of *FGFR3*, reflecting the low percentage of mutations in

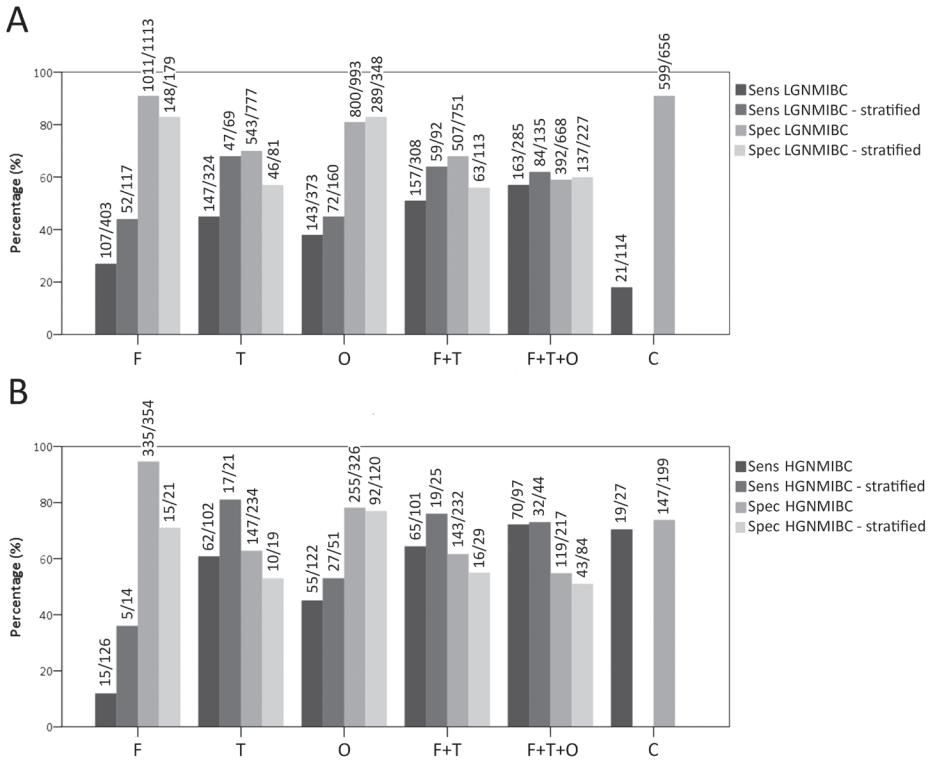

Figure 6.2 *Marker performance for recurrence detection in patients with primary (A) low-grade and (B) high-grade NMIBC: unstratified and stratified. F=FGFR3 analysis, T=TERT analysis, O=OTX1 analysis, C=cytology.*

these tumors.[13] The 3-assay combination identified 72% (70/97) of all recurrences (Figure 6.2b). Again sensitivity increased with higher stage and grade of the recurrences (Table 6.3).

In order to get a realistic view of marker sensitivity we stratified patients based on the molecular analysis of tumor DNA. There was tumor DNA available in 342 NMIBC patients: 239 LG-NMIBC and 103 HG-NMIBC. *FGFR3* and *TERT* mutations were found in 69% and 77% of LG-NMIBC and in 26% and 58% of HG-NMIBC, respectively. 88% of the LG-NMIBC and 67% of the HG-NMIBC was mutant for either *FGFR3* or *TERT*. *OTX* methylation was observed in 93% of LG-NMIBC and 91% of HG-NMIBC. A total of 176 tumors were analyzed for all three assays of which 4 (2%) tumors did not harbor any alteration, 29 (16%) had one mutation or methylation, two aberrations were found in 71 (41%) tumors and all three were present in 72 (41%). Stratification was based on the molecular status of the analyzed tumor, assuming that the molecular make-up of the recurrent tumors was similar. This resulted in an improvement of sensitivity for all markers. In 44% (52/117) of the LG-NMIBC patients with a *FGFR3* mutant tumor, the recurrence was detected by *FGFR3* urine analysis (Figure 6.2a). Likewise, 45%

Table 6.2a: Tumor stage of detected and missed recurrences from patients with primary low-grade NMIBC by the combination of FGFR3, TERT, and OTX1 analysis.

	Ta	T1	T2-4	Tx	pTis	Total
All negative	102	2	3	12	3	122
	44,9%	12,5%	23,1%	50,0%	60,0%	42,8%
≥1 positive	125	14	10	12	2	163
	55,1%	87,5%	76,9%	50,0%	40,0%	57,2%
Total	227	16	13	24	5	285

Table 6.2b: Tumor grade of detected and missed recurrences from patients with primary low-grade NMIBC by the combination of *FGFR3, TERT,* and *OTX1* analysis.

	G1	G2	G3	Gx	PUNLMP	LG	HG	CIS	Total
All negative	32	23	3	11	8	35	7	3	122
	49,2%	42,6%	16,7%	44,0%	72,7%	37,2%	50,0%	75,0%	42,8%
≥1 positive	33	31	15	14	3	59	7	1	163
	50,8%	57,4%	83,3%	56,0%	27,3%	62,8%	50,0%	25,0%	57,2%
Total	65	54	18	25	11	94	14	4	285

Table 6.3a: Tumor stage of detected and missed recurrences from patients with primary high-grade NMIBC by the combination of *FGFR3*, *TERT*, and *OTX1* analysis.

	Ta	T1	T2-4	Tx	pTis	Total
All negative	12	7	4	3	1	27
	30,8%	31,8%	28,6%	30,0%	8,3%	27,8%
≥1 positive	27	15	10	7	11	70
	69,2%	68,2%	71,4%	70,0%	91,7%	72,2%
Total	39	22	14	10	12	97

Table 6.3b: Tumor grade of detected and missed recurrences from patients with primary high-grade NMIBC by the combination of ***FGFR3, TERT,*** and ***OTX1*** analysis.

	G1	G2	G3	Gx	PUNLMP	LG	HG	CIS	Total
All negative	1	2	4	0	6	9	4	1	27
	20,0%	22,2%	17,4%	0,0%	50,0%	52,9%	22,2%	8,3%	27,8%
≥1 positive	4	7	19	1	6	8	14	11	70
	80,0%	77,8%	82,6%	100,0%	50,0%	47,1%	77,8%	91,7%	72,2%
Total	5	9	23	1	12	17	18	12	97

(72/160) of the recurrences were detected by *OTX1* methylation analysis in patients with tumor methylation of the *OTX1* gene. Stratification based on the molecular status of the tumor was most beneficial in patients with primary LG-NMIBC and a *TERT* mutation. In these patients the rate of recurrence detection by *HTERT* urine analysis increased from 46 to 68%. Stratification based on all three markers slightly increased the sensitivity for recurrence detection in patients

with primary LG-NMIBC from 57% to 62%. This was to be expected because one of the three markers is altered in 98% of the tumors. Similar stratification results were found in patients with a primary HG-NMIBC (Figure 6.2b). We conclude that stratification increases sensitivity for the individual markers but not much for the combination (Figure 6.2).

Next, we investigated whether there was a difference in marker performance between males and females. Interestingly, a significantly higher sensitivity for all (combination of) markers was observed in male compared to female LG-NMIBC patients, except for *OTX* (p=0.168). The 3-assay combination detected 63% of the recurrences from the male patients, compared to 44% of the recurrent tumors from the female patients (p=0.003) (Figure 6.3a). A possible explanation for these findings is the presence of more normal cells, such as vaginal cells, in urine from women. To support this hypothesis, we analyzed urine DNA yield as a surrogate for the number of cells and we indeed found significantly higher DNA concentrations in urine samples from female patients compared to male patients (15.5ng/µl vs. 11.4ng/µl, p<0.001). Differences in marker performance between males and females were less pronounced in the HG-NMIBC patients (Figure 6.3b) and although urine from females yielded more DNA, this difference in DNA concentration was not significant (19.35ng/µl vs. 15.25ng/µl, p=0.083).

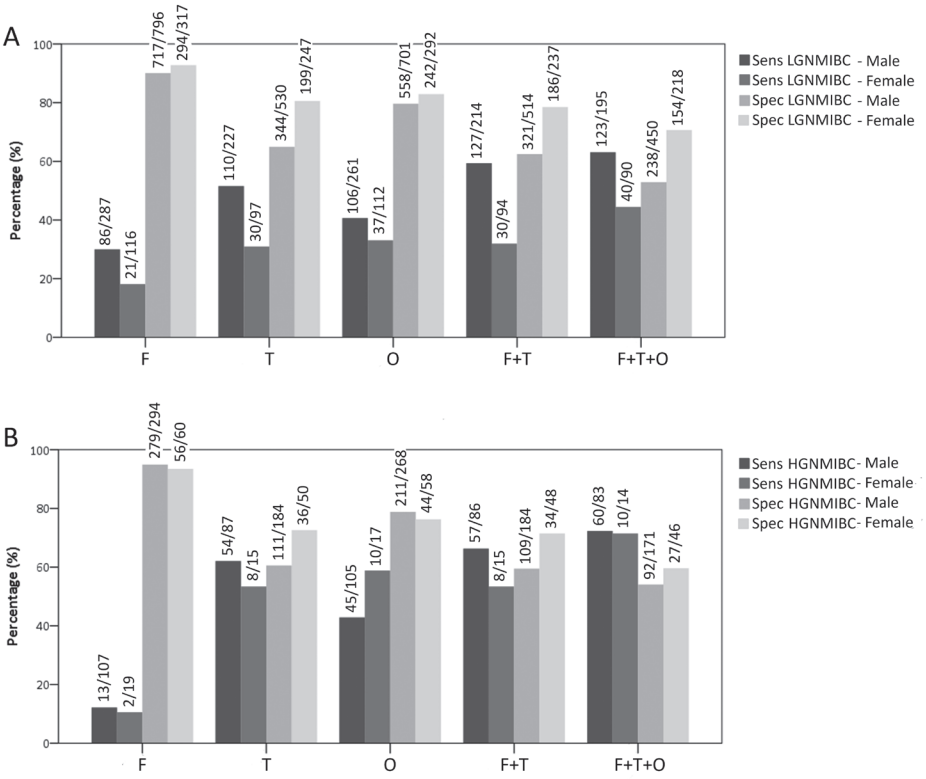

Figure 6.3 *Marker performance for recurrence detection in patients with primary (A) low-grade and (B) high-grade NMIBC: Male vs. Female. F=FGFR3 analysis, T=TERT analysis, O=OTX1 analysis.*

In total 318 urine samples collected at first diagnosis were available for analysis. This included 230 samples associated with a primary LG-NMIBC and 87 samples with a HG-NMIBC. For all three assays, the sensitivity for detection of a primary HG-NMIBC or LG-NMIBC was much higher compared to the sensitivity for recurrence detection. The combination of three assays detected 81% (113/140) of all primary LG-NMIBC and 94% (61/65) of the primary HG-NMIBC. Again, detection rate increased with higher stage and grade of the primary tumor (supplementary table 6.4 and 6.5).

Overall, relatively high false-positives rates were observed for concomitant recurrence detection. We believe this is partly due to the anticipatory effect of urine analysis, meaning that the urinary assay detects the tumor before it is visible by cystoscopy. To show this effect, every first positive follow-up urine sample of a patient was selected and plotted against time to next recurrence. Importantly, 86% of the FGFR3-positive urine samples were followed by a recurrence within 5 years (Figure 6.4a). Likewise, in 78% of the urines samples positive for the *TERT* mutation a recurrence occurred within a period of 5 years and in 76% of the urine samples positive for the 3-assay combination (Figure 6.4b and 6.4c). Subsequently, we investigated how recurrences of different stages were detected in time after a positive urine test. Interestingly, high stage recurrences were mostly identified within the first year after urine analysis, whereas stage Ta recurrences were continuing to be observed for a long time after a positive urine test.

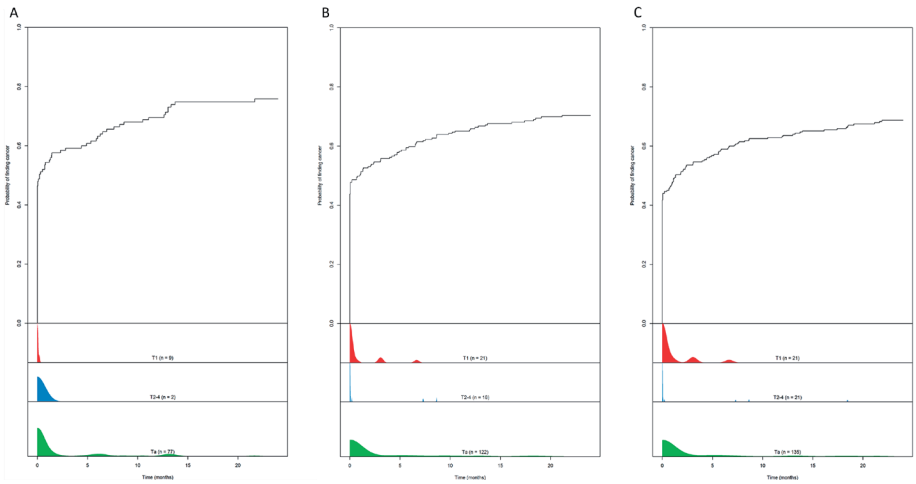

Figure 6.4 *Positive urine samples followed by recurrence over time: 86% of all FGFR3 positive samples(A), 78% of all TERT positive samples (B), and 76% of all urine samples positive for FGFR3 mutation and/or TERT mutation and/or OTX methylation (C) were followed by recurrence within 5 years.*

These findings underline the additional value of urinary markers for earlier detection of high-risk tumors.

DISCUSSION

In this study we evaluated the performance of three assays for the detection of recurrent BC in voided urine, i.e. *FGFR3* mutation analysis, *TERT* mutation analysis and the *OTX* methylation assay, especially in patients with primary low-grade disease. Previously, these assays proved to be sensitive for the detection of recurrent bladder tumors in smaller patient series.[9, 15, 16] This is the first study on urine analysis involving a large multicenter prospective European patient cohort.

All assays were superior to urine cytology for recurrence detection in patients with primary LG-NMIBC. Combination of the three assays improved the recurrence detection rate to 57%. Overall, most of the aggressive recurrences in patients with a primary LG-NMIBC were detected by this combination with a sensitivity of 77% for MI recurrences, and 83% for G3. Remarkably, we found higher marker performance in male patients with primary LG-NMIBC compared to female patients, suggesting the disturbing influence of the high amount of benign cells in female urine. This difference was not observed in the HG-NMIBC patients. This could be due to the higher amount of tumor cells shed into urine in the high-grade tumors, leading to less disturbing influence of the benign cells. Furthermore, we showed that almost all positive urine samples were followed by a recurrence over time and all high-stage recurrence were identified within 1 year of follow-up. We believe that this is a very important finding, since it could contribute to earlier detection of aggressive tumors.

FGFR3 mutation analysis after stratification based on the mutation status of analyzed tumor resulted in lower marker sensitivity compared to previous studies.[9, 17] This could be caused by the larger prospective patient population with a higher amount of LG-NMIBC recurrences. Overall sensitivity of 46% for the combination of *FGFR3* and *TERT* mutation analysis for the detection of recurrences in primary LG-NMIBC patients was similar to previously reported data.[15] Previously, Kandimalla *et al* reported a sensitivity of 65% for the *OTX* methylation analysis with a specificity of 90%.[16] The negative controls included in that study were collected from healthy men. In our study, all samples were derived from patients with a history of bladder cancer. We observed that, in patients of the current study that already had bladder cancer, a higher background of methylation was measured. Therefore, we decided to use a higher cut-off for the methylation ratio of *OTX* allowing 20% false-positives. Subsequently, this resulted in a lower sensitivity than reported previously. The three-assay combination was significantly better for the detection of recurrences in patients with primary HG-NMIBC compared to patients with primary LG-NMIBC (sensitivity 72% vs. 57%, p=0.009). Likewise, higher detection rates were observed in urine samples collected prior the resection of the initial tumor compared to follow-up urine samples. This again emphasizes the difficulties in the development of a sensitive urine test for the detection of low-grade recurrences. As previously mentioned, these tumors are mostly smaller in size compared to the original primary tumor. Small tumor size together with low tumor grade will result in a reduced shedding of tumor cells. Therefore, the amount of tumor DNA is often below the analytical threshold leading to false-negative test outcomes. Multiple urine testing could minimize this problem as shown in a previous study.[18]

Overall, specificity of the combination of three assays was relatively low. Several reasons could be given for the high false-positive rate of urine analyses. First, often not realized, the diagnostic ability of the cystoscopy is not flawless. Up to 29% of the Ta tumors and 25% of the T1 tumors are missed by white-light cystoscopy.[3] Therefore, in several patients with 'false-positive' urine samples there could have been a tumor present, yet it was missed by cystoscopy. Secondly, positive urine assays are often followed by a positive cystoscopy later in time, the so-called anticipatory effect. The specificity of *FGFR3* mutation analysis is generally considered to be 100%, since these mutations are only found in sufficient amounts in tumor cells.[19] We showed that 86% of the *FGFR3* positive urine samples were followed by a recurrence within 5 years. Likewise, we found that 76% of the urine samples positive for the 3-assay combination were associated with a recurrence within 5 years. Thirdly, small recurrences are often coagulated without collecting tissue for pathological examine. Since we defined recurrence as a histologically proven tumor, we did not include these coagulated recurrences in the data analysis. Finally, a small proportion of recurrences will appear as upper urinary tract tumor that are be missed by cystoscopy. This data was not available in this study. in the data analysis.

Strengths of the study include the multicenter prospective character and the analysis of a substantial number of follow-up urine samples, including multiple samples per patient. One of the limitations was the low number of urine samples that could be analyzed for *TERT* mutations, since the study had already started when this assay was under construction.

CONCLUSIONS

This is the first study on urine analysis in mainly LG-NMIBC patients including a large prospective European multicenter patient cohort. We showed that a combination of urinary assays increased sensitivity for recurrence detection in low-risk patients and assume that sensitivity will further increase by testing of multiple urine samples per patient. The combination detected most concomitant aggressive tumors. Furthermore, we observed that a positive urine assay was almost always followed by a recurrence. This study supports the value of urine analysis as an alternative for cystoscopies in patients with LG-NMIBC and as a valuable addition to the surveillance of patients presenting with HG tumors. Next step in investigating the role of urine analyses in the follow-up of NMIBC patients would be a randomized control trial, which is currently in progress. As future perspective, we advocate a urine first-strategy, meaning that the analysis of urine samples will reduce the number of cystoscopies, since only a positive urine test will be followed by cystoscopy. This will be mostly relevant for patients with low-risk Ta-tumors, especially because the sensitivity of cystoscopy is relatively low for the detection of these Ta-tumors.

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SUPPLEMENTARY FILES

FGFR3 mutation analysis

The FGFR3 analysis was performed as described previously by van Oers et al.[1] The FGFR3 assay consists of a multiplex PCR comprising the three regions with most frequent mutations, i.e. Exon 7 (R248C and S249C), exon 10 (G372C, S737C, Y375C, G382R and A393E) and 15 (K652M, K652T, K652E K652Q). Primers sequences are depicted in supplementary table 6.1a. PCR was carried out in 15µl containing 5ng of DNA, 1x GO-Taq PCR buffer (Promega), 1.5mmol/l $MgCl_2$, 0.17mmol/l deoxynucleotide triphosphates (Roche), 1.0 unit Taq polymerase (Promega), 5% Glycerol (Fluka), 18 pmol of exon 7 primers and 10 pmol of exon 10 and 15 primers. PCR Cycling started at 95°C for 3 minutes followed by 40 cycles at 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 2 seconds. Next, samples stayed at 72°C for 10 minutes. The PCR product was treated with 3 units of shrimp alkaline phosphatase (Amersham bioscience) and 2 units of exonuclease-I (Amersham bioscience) and incubated for 60 minutes at 37°C followed by 15 minutes at 72°C, in order to remove the overload of primers and deoxynucleotide triphosphates. This was followed by a single-nucleotide probe extension assay using the SNaPshot Multiplex kit (Applied Biosystems) in combination with probes that anneal directly next to investigating mutation site. Different lengths of T-tail were attached to the probes at their 5′ end allowing separation of the products based on size. Probe sequences and concentrations are given in supplementary table 6.1b. The SNaPshot reaction was carried out in a total volume of 10µl comprising 1µl of PCR product, 2.5µl of the SNaPshot ready mix, 2µl of the 5x sequence buffer, and 1µl of the probemix. Thermal cycling conditions were 35 cycles of 10 seconds at 96°C, followed by 40 seconds at 58.5°C. As final step the SNaPshot product was treated with 1 unit of shrimp alkaline phosphatase to remove the excess of dideoxynucleotide triphosphates (ddNTPs). The final product was separated on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the fluorescent label on the incorporated ddNTP indicating the absence or presence of a mutation.

TERT mutation analysis

The *TERT* mutation analysis was performed as described previously by Allory et al.[2] and is similar to the FGFR3 mutation analysis. The PCR covered the two most frequent mutation sites (G228A/T and G250A). Primer and probe sequences are given in supplementary table 6.2. All experimental conditions were equal to the *FGFR3* analysis.

Methylation analysis

Methylation analysis, also known as the Bisulphite Specific-SnaPhot, was done as described by Kandimalla et al.[3] The assay includes bisulphite conversion of the genomic DNA (EZ DNA methylation gold kit, Zymo Research Corp). The following steps are similar to the mutation analyses and involve amplification of the region with the CpG island from the gene of interest and single nucleotide probe extension using the SNaPshot Multiplex kit. Primer and probe sequences are

given in supplementary table 6.3. The fluorescent label on the incorporated ddNTP indicated the presence or absence of a methylation of the investigated CpG.

Supplementary table 6.1a: primer sequences and conditions *FGFR3* mutation analysis

Primer	Sequence (5'->3')	Product size (bp)	pmol per reaction
FGFR3 RI Fw	AGTGGCGGTGGTGGTGAGGGAG	115	18
FGFR3 RI Rev	GCACCGCCGTCTGGTTGG		18
FGFR3 RII Fw	CAACGCCCATGTCTTTGCAG	138	10
FGFR3 RII Rev	AGGCGGCAGAGCGTCACAG		10
FGFR3 RIII Fw	GACCGAGGACAACGTGATG	160	10
FGFR3 RIII Rev	GTGTGGGAAGGCGGTGTTG		10

Supplementary table 6.1b: probe sequences and conditions *FGFR3* mutation analysis

Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	pmol per reaction
S373C	T19 GAGGATGCCTGCATACACAC	39	sense	T	A	2
K652M/T	T20 CACAACCTCGACTACTACAAGA	42	sense	A	T/C	7
G372C	T29 GGTGGAGGCTGACGAGGCG	48	sense	G	T	2
A393E	T34 CCTGTTCATCCTGGTGGTGG	54	sense	C	A	10
R248C	T46 CGTCATCTGCCCCCACAGAG	66	sense	C	T	8
Y375C	T43 ACGAGGCGGGCAGTGTGT	61	sense	A	G	10
S249C	T36TCTGCCCCCACAGAGCGCT	55	sense	C	G	4
K652Q/E	T50 GCACAACCTCGACTACTACAAG	72	antisense	A	C/G	3
G382R	T56 GAACAGGAAGAAGCCCACACC	76	antisense	C	T	6

Supplementary table 6.2a: primer sequences and conditions *hTERT* mutation analysis

Primer	Sequence (5'->3')	Product size (bp)	pmol per reaction
hTERT Fw	AGCGCTGCCTGAAACTCG	155	10
hTERT Rev	CCCTTCACCTTCCAGCTC		10

Supplementary table 6.2b: probe sequences *hTERT* mutation analysis

Probe	Sequence (5'->3')	Size (bp)	Strand	WT	MT	pmol per reaction
hTERT 1295228	T20 GGCTGGGAGGGCCCGGA	37	sense	G	A/T	10
hTERT 1295242	T27 GGAGGGGGCTGGGCCGG	44	sense	G	A	5
hTERT 1295250	T39 CTGGGCCGGGGACCCGG	56	sense	G	A	15

Supplementary table 6.3: primer sequences and conditions *OTX* methylation analysis

Primer/probe	Sequence (5'->3')	Product size (bp)	Meth	Unmeth	pmol per reaction
OTX1 Fw	TTTTGAGAGGTATAGAGAGGGGTAGT	172			10
OTX1 Rv	CCCCTAACAAACCCAAATCTC				
SNaPshot probe *OTX1*	TTTATTTGTGGTTTTTTAGGTT	23	C	T	5

Supplementary table 6.4: Tumor stage of detected and missed primary tumors from patients with primary low-grade NMIBC by the combination of FGFR3, TERT, and OTX1 analysis.

	Ta	T1	Total
All negative	25	2	27
	19,8%	14,3%	19,3%
At least 1 positive	101	12	113
	80,2%	85,7%	80,7%
Total	126	14	140

Supplementary table 6.5: Tumor stage of detected and missed primary tumors from patients with primary high-grade NMIBC by the combination of FGFR3, TERT, and OTX1 analysis.

	Ta	T1	Tis	Total
All negative	2	2	0	4
	9,5%	4,9%	0,0%	6,2%
At least 1 positive	19	39	3	61
	90,5%	95,1%	100,0%	93,8%
Total	21	41	3	65

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Chapter 7

The use of molecular analyses in voided urine for the assessment of patients with hematuria

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ABSTRACT

Objective | Patients presenting with painless hematuria form a large part of the urological patient population. In many cases, especially in younger patients, the cause of hematuria is harmless. Nonetheless, hematuria could be a symptom of malignant disease and hence most patients will be subject to cystoscopy. In this study, we aimed to develop a prediction model based on methylation markers in combination with clinical variables, in order to stratify patients with high risk for bladder cancer.

Material and methods | A total of 169 patients presenting with painless hematuria were included. 54 patients were diagnosed with bladder cancer. In the remaining 115 patients, the cause of hematuria was non-malignant. Urine samples were collected prior to cystoscopy. Urine DNA was analyzed for methylation of *OSR1*, *SIM2*, *OTX1*, *MEIS1* and *ONECUT2*. Methylation percentages were calculated and were combined with clinical variables into a logistic regression model.

Results | Logistic regression analysis based on the five methylation markers, age, gender and type of hematuria resulted in an area under the curve (AUC) of 0.88 and an optimism corrected AUC of 0.84 after internal validation by bootstrapping. Using a cut-off value of 0.307 allowed stratification of patients in a low-risk and high-risk group, resulting in a sensitivity of 82% (44/54) and a specificity of 82% (94/115). Most aggressive tumors were found in patients in the high-risk group. The addition of cytology to the prediction model, improved the AUC from 0.88 to 0.89, with a sensitivity and specificity of 85% (39/46) and 87% (80/92), retrospectively.

Conclusions | This newly developed prediction model could be a helpful tool in risk stratification of patients presenting with painless hematuria. Accurate risk prediction might result in less extensive examination of low risk patients and thereby, reducing patient burden and costs. Further validation in a large prospective patient cohort is necessary to prove the true clinical value of this model.

INTRODUCTION

Hematuria is one of the most common symptoms in urological practice, as up to 20% of all urological visits are for hematuria[1]. Hematuria in the adult population can have different causes, e.g. urinary tract infections, urolithiasis, benign prostate enlargement (BPH), and urologic malignancies. A urological cancer is found in approximately 5% of patients presenting with microscopic hematuria and in around 20% of patients with macroscopic hematuria[2,3]. However, in up to 60% of the patients no source of bleeding is found. Thus, most important in the evaluation of hematuria is the discrimination between a malignant and non-malignant cause. As a consequence, patients will be subject to an extensive examination, including cystoscopy, cytology and imaging of the upper urinary tract. The sensitivity of cystoscopy for the detection of bladder cancer (BC) is high, ranging from 68-83%[4,5]. Yet, it is an invasive procedure, causing pain and discomfort. Cytology has a high specificity, but a poor sensitivity especially for the detection of low-grade BC. Therefore cytology is only used in combination with cystoscopy.

The use of molecular markers in the assessment of hematuria could be of importance in order to reduce costs and to avoid invasive diagnostic procedures. However, none of the current investigated markers have a high enough sensitivity and specificity to accurately distinguish between malignant and non-malignant causes.

Recently, we developed a combination of methylation markers for urine-based follow-up of bladder cancer patients. The sensitivity of the combination of markers was 74% for the detection of bladder cancer recurrences[6]. The aim of the current study is to investigate whether these markers could also be used to predict the risk of urothelial cell carcinoma (UCC) in patients presenting with painless microscopic and macroscopic hematuria.

MATERIAL AND METHODS

Patients and urine samples

In this study, urine samples were included from patients presenting with painless microscopic or macroscopic hematuria. All urine samples were retrieved from a sample bank. This sample bank contained urine samples that were prospectively collected prior to cystoscopy between January 2007 and July 2012 at the Urology outpatient departments of Erasmus MC, Rotterdam and Aarhus University Hospital, Denmark. There were 54 urine samples available from which the cause of hematuria was malignant. As control group we included 115 hematuria samples with a non-malignant cause.

All patients were examined by cystoscopy, computer tomography of the abdomen, and renal ultrasound. In addition, urine cytology was performed. Bladder tumors were biopsied and confirmed by histology. Urine samples were collected prior cystoscopy and were processed within 12-hours after collection. Urine samples were centrifuged for 10 minutes at 2000*g. Cell pellets were re-suspended in 1ml PBS and centrifuged for minutes at 3000*g. Supernatant was

discarded and cell pellets were stored at -80°C until DNA isolation. DNA was extracted using the QIAamp mini and Blood kit (Qiagen) according to manufacturer's protocol. Samples from Erasmus MC were used according to the code of secondary use of human tissue (www.federa.org). All patients were checked in the Erasmus MC opt-out objection system. In case of objection, patients were excluded from analysis. For minors, parents or caretakers should have filled in the statement of objection. Informed written consent was obtained from patients at Aarhus University Hospital and the study was approved by the Central Denmark Region Committees on Biomedical Research Ethics (1994/2920).

Methylation analysis

Recently, we developed a multiplex methylation assay in order to detect bladder cancer in voided urine[6]. This assay consisted of probes covering CpG-sites in five different genes, namely *OSR1, OTX1, ONECUT2, MEIS1* and *SIM*. Methylation analysis was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, California, USA) according to the manufacturer's protocol. Briefly, DNA was treated with sodium bisulfite, followed by bisulfite-specific PCR for the five regions of interest. For each PCR reaction a DNA input of 20ng and PCR primer concentration of 20 pM was required. After the PCR, a Single Nucleotide Primer Extension (SNuPE) analysis was performed, using primers that annealed to the PCR product adjacent to the cytosine of interest. SNuPE probes were extended with a labeled dideoxynucleotide and the products were analyzed on an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a methylated cytosine. See Kandimalla *et al* for details on the primers and probes used[6]**.** For each gene, the methylation percentage was calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peaks multiplied by hundred.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences 20 (SPSS, Chicago) and R statistical Software for Statistical Computing (Vienna).

Univariable and multivariable logistic regression models were used to calculate the association between UCC and the predictor variables. The predictive accuracy of the model was determined by the area under the curve (AUC). The Bootstrap procedure was used for internal validation of the predictive model. P-values <0.05 were considered statistically significant.

RESULTS

A total of 169 patients with painless microscopic or macroscopic hematuria were included in this study, comprising 104 men and 65 women. The mean age was 59 years (range 17-92). Patients and tumor characteristics are depicted in Table 7.1. In 54 cases, a bladder tumor was the cause of hematuria. In the remaining 115 patients, the cause of hematuria was non-malignant.

Table 7.1: Clinical and histopathological characteristics of 169 patients presenting with painless hematuria

		Hematuria UCC+	Hematuria UCC-
		n=54	n=115
Age	*Mean (range)*	67 (29-92)	55 (17-86)
		n (%)	n (%)
Sex	*Male*	37 (69)	67 (58)
	Female	17 (31)	48 (42)
Hematuria	*microscopic*	18 (33)	68 (59)
	macroscopic	36 (67)	47 (41)
Cytology	*no tumor cells*	28 (52)	89 (77)
	tumor cells	18 (33)	3 (3)
	not performed	8 (15)	23 (20)
Stage	*Ta*	30 (55)	
	T1	7 (13)	
	>=T2	13 (24)	
	Tx	3 (6)	
	Tis	1 (2)	
Grade			
WHO1973	*G1*	6 (11)	
	G2	11 (20)	
	G3	19 (35)	
	Gx	4 (7,5)	
WHO2004	*Low Grade*	10 (19)	
	High Grade	4 (7,5)	

Patients with UCC were significantly older and presented more frequent with macroscopic hematuria compared to patients with a non-malignant cause (p<0.001 and p=0.002). Cytology was performed in 81% of the patients. The sensitivity and specificity for cytology were 39% and 97, retrospectively.

Methylation analysis was performed for *OSR1*, *SIM2*, *OTX1*, *MEIS1* and *ONECUT2* and methylation percentages were calculated. Next, univariable logistic regression analysis was performed for the methylation percentages of the five different genes and the clinical variables age, gender, type of hematuria and cytology (Table 7.2). *OSR1*, *SIM2*, *OTX1*, *MEIS1, ONECUT2*, age, type of hematuria and cytology were all significant predictors for the presence of UCC. In order to calculate the combined effect, multivariable logistic regression analysis was performed. First a model was developed, based on the methylation percentages of the five genes and the clinical variables age, gender and type of hematuria. This resulted in an apparent AUC of 0.88 (Figure 7.1). In this model age, type of hematuria, *ONECUT2*, *OSR1* and *SIM2* were independent predictors for the presence of UCC (Table 7.3). After internal validation by bootstrapping, the optimism-corrected AUC was 0.84. To discriminate between patients at low-risk for UCC vs. patients at

Table 7.2: Univariable logistic regression analyses assessing the association between predictors and the presence of urothelial cell carcinoma

	OR	95% CI	p value	AUC
Age (continuous)	1.062	(1.033, 1.092)	<0.001	0.72
Gender	0.641	(0.324, 1.270)	0.203	0.55
Type of hematuria	2.894	(1.033, 1.092)	0,002	0,63
Cytology	19.071	(5.229, 69.555)	<0.001	0.68
OTX ratio	1.061	(1.031, 1.093)	<0.001	0.69
ONECUT ratio	1.096	(1.050, 1.144)	<0.001	0.78
OSR ratio	1.072	(1.041, 1.103)	<0.001	0.75
SIM ratio	1.043	(1.021, 1.066)	<0.001	0.60
MEIS ratio	1.069	(1.034, 1.105)	<0.001	0.71

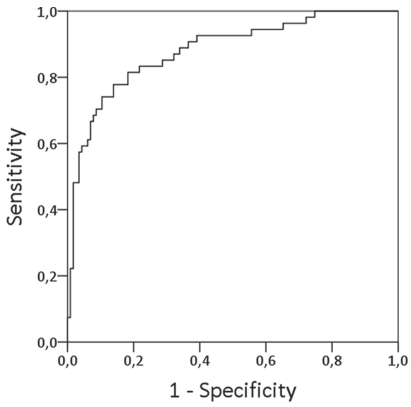

Figure 7.1 *ROC of the multivariable model (AUC 0.88)*

high-risk for UCC, a cut-off value with optimal sensitivity and specificity was determined. Based on a cut-off of 0.307, 44/54 of patients with UCC were in the high-risk group, resulting in a sensitivity of 82%. 94/115 (82%) of patients without a malignancy were in the low-risk group. In figure 7.2, the predictive values are shown according to stage (Fig 7.2A) and grade (Fig 7.2B) of the detected tumors. Tumors that were detected in patients in the low-risk group were mostly low stage and low grade. However, there was also one patient with a grade 3 tumor. On the other hand, all patients with a ≥pT2 tumor were in the high-risk group. In order to determine the additional value of cytology, a second model was developed. This model resulted in an AUC of 0.89 as shown in table 7.3 and an optimism-corrected AUC of 0.85. Based on a cut-off of 0.306, the sensitivity and specificity for the second model were 85% (39/46) and 87% (80/92), respectively.

Table 7.3: Multivariable logistic regression analyses assessing the association between predictors and the presence of urothelial cell carcinoma

Multivariable model 1	OR	95% CI	p value	AUC (%)
Age (continuous)	1.047	1.014, 1.081	0.005	88.1
Gender	0.924	0.376, 2.275	0.864	
Type of hematuria	3.743	1.521, 9.212	0.004	
OTX ratio	1.038	0.936, 1.152	0.478	
ONECUT ratio	1.117	1.015, 1.228	0.024	
OSR ratio	1.010	1.010, 1.124	0.020	
SIM ratio	0.829	0.829, 0.980	0.015	
MEIS ratio	0.986	0.919, 1.057	0.686	
Multivariable model 2 (including cytology)	**OR**	**95% CI**	**p value**	**AUC (%)**
Age (continuous)	1.032	0.997, 1.069	0.077	89.5
Gender	0.693	0.242, 1.989	0.496	
Type of hematuria	2.347	0.866, 6.361	0.094	
Cytology	10.956	2.269, 52.918	0.003	
OTX ratio	1.053	0.938, 1.181	0.384	
ONECUT ratio	1.085	0.981, 1.201	0.113	
OSR ratio	1.080	1.018, 1.145	0.010	
SIM ratio	0.875	0.786, 0.973	0.014	
MEIS ratio	0.988	0.907, 1.076	0.784	

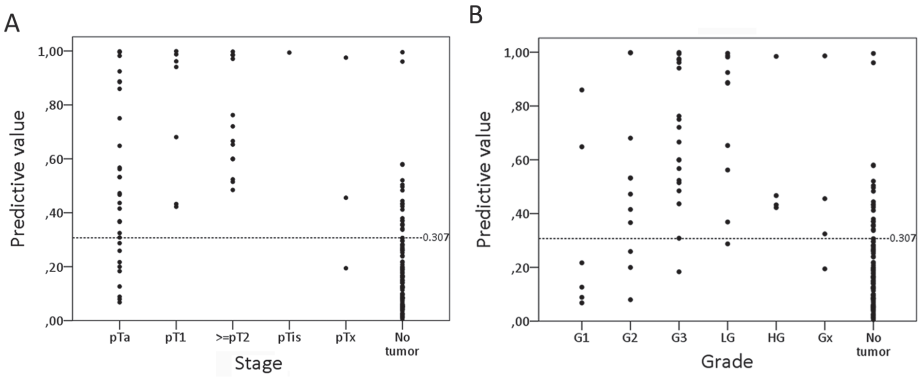

Figure 7.2 Scatterplot of risk values for the detection of urothelial cell carcinoma (UCC) in patients presenting with painless hematuria: X-axis depicts stage (A) and grade (B) of the resected tumors. Y-axis depicts the risk value based on the developed logistic regression model. Patients with risk value > 0.307 were considered at high-risk for having UCC. Patients with a risk value < 0.307 were considered at low-risk. Each dot represents a urine sample.

DISCUSSION

Painless hematuria is a major problem in urological practice and the distinction between malignant and non-malignant causes is crucial. In this study, we developed a prediction model for the assessment of patients presenting with painless microscopic or macroscopic hematuria. With this newly developed model, the urologist will be able to adjust patient examination according to patient risk, resulting in a reduction of costs and patients discomfort. Previously, the five methylation markers were proven to be sensitive for the detection of recurrent UCC[6]. The methylation assay also appeared highly reproducible between different investigators. In the current study, the five markers discriminated with a high sensitivity between patients with and those without primary bladder cancer. Addition of the clinical variables age, gender and type of hematuria increased the accuracy of the model. Age is one of the greatest risk factors for the development of bladder cancer and since men have a 3-4 times higher chance of developing UCC compared to women, gender also contributes significantly[7]. The use of cytology even more improved the diagnostic accuracy. Yet, we believe that the use of cytology as diagnostic test especially for the detection of low grade tumors is debatable. Therefore, we decided to calculate the first prediction model without the addition of cytology. Inclusion of smoking history may even improve the predictive value further, since previous studies demonstrated that a history of smoking is an important independent predictor in the evaluation of hematuria[7-9]. However, smoking history was unavailable for the patients in this study.

Up to now, multiple studies have been performed on the use of molecular tests in the diagnosis of bladder cancer in patients presenting with hematuria and some of these assays are already FDA approved[10-13]. However, due to suboptimal sensitivities and specificities, the analyses are mostly performed in addition to cystoscopy. Abogunrin et al. investigated whether biomarkers were able to improve the predictive power of a risk model which was based on clinical variables[14]. They considered the additional predictive value of nine different biomarkers to the prior predictive probability (PPP) that was based on age and smoking. They concluded that the addition of nuclear matrix protein 22 and vascular endothelial growth factor to the PPP improved the diagnostic accuracy from 0.76 to 0.90. In another study by Cha et al, the authors also combined molecular tests and clinical features into a multivariable regression model in order to predict the likelihood of having bladder cancer. They developed a nomogram based on the commercially available immunocytology assay (uCyt/ImmunoCyt), in combination with conventional cytology and clinical variables, i.e. age, gender, smoker, hematuria (microscopic vs. macroscopic)[15]. The AUC of this multivariable model was 0.904. However, cytology and immunocytology are highly dependent on the skills and experience of the pathologist[16].

A limitation of the current study was the retrospective design. Since this was not a consecutive series of patients, the composition of the patient cohort does not reflect true clinical practice. In addition, this model was internally validated by using the bootstrap method[17]. Therefore we suggest this model should be externally validated in a large prospective patient cohort of patients presenting with painless microscopic or macroscopic hematuria.

CONCLUSIONS

We developed an accurate risk model for the evaluation of patients presenting with painless hematuria. Predicting the risk of bladder cancer in these patients could be of great value, resulting in less extensive examination of low risk patients and reduction of costs. Further validation in a large prospective patient cohort is necessary to prove the true clinical value of this newly developed model.

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PART IV

EPIGENETIC AND GENETIC ALTERATIONS IN YOUNG BLADDER CANCER PATIENTS

Chapter 8

Hypermethylation of the polycomb group target gene *PCDH7* in bladder tumors from patients of all ages

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ABSTRACT

Objective | Bladder tumors of patients <20yr have a low incidence of genetic and epigenetic aberrations typically found in older patients. One of the most common epigenetic aberrations in human malignancies is DNA hypermethylation. Polycomb group (PcG) complexes play an important role during lineage choices in embryogenesis and their target genes are 12 times more likely to be methylated than non-PcG target genes. We hypothesized that methylation of PcG target genes is an early event in urothelial carcinogenesis and thus might be observed in young patients.

Material and methods | Patients (n=167) were stratified according to age into four groups: <20yr (n=14), 20-40yr (n=48), 40-60yr (n=47) and >60yr (n=58). Five PcG target genes identified by Kandimalla *et al* (*MEIS1, ONECUT2, OTX1, PCDH7* and *SOX21*) were selected for methylation analysis. Methylation ratios were calculated by using the unmethylated and methylated signal. The outcome represented the fraction of methylated cells within one tumor. Genes with similar methylation ratios in all age groups were considered as potential bladder cancer initiating candidates.

Results | Three genes showed higher methylation ratios in tumors from older patients*: ONECUT2* ($p<0.001$), *SOX21* ($p<0.001$) and *OTX1* ($p<0.001$). *MEIS1* displayed similar methylation ratios in all groups. However the median methylation ratio was low. *PCDH7* exhibited similar median methylation percentages in all age categories, i.e. <20yr 54%; 20-40yr 59%; 40-60yr 59%; >60yr 67% ($p=0.1$).

Conclusions | Tumors from young patients showed less methylation for most markers. *PDH7* showed high methylation ratios in all age categories and could therefore play an important role in early urothelial carcinogenesis.

INTRODUCTION

Bladder cancer (BC) is a disease of the elderly with a peak incidence in the sixth decade of life and only 1-2.4% of all cases are under the age of 40.[1-3] BC in patients younger than 20 years is even more uncommon with reported incidence rates of only 0.1-0.4%.[2, 4] Conflicting results have been published regarding clinical outcome of these young patients. Some studies have observed a similar disease course in both young and older patients,[2, 5] while other studies have reported a more favorable clinical outcome in younger patients with less recurrences and disease progression.[1, 6-8] This discrepancy may be caused by the wide variation in the definition of a young patient; this ranges from <20 years to <40 years.

Patients with BC <20 years have mainly been described in smaller studies, including case reports and therefore bladder carcinogenesis in young patients is not well defined. It is still unclear whether BC arising in younger patients proceeds through the same molecular pathways as those seen in their older counterparts. Interestingly, tumors of patients <20 years are predominantly of low stage and grade with a lack or much lower incidence of epigenetic and genetic aberrations typical of bladder cancer in elderly patients.[7, 9, 10] Since BC patients <20yr seem to be a biologically distinct group, we decided to investigate the underlying mechanism of bladder carcinogenesis in young patients.

Genetic and epigenetic aberrations play an important role in the formation of many carcinomas. The most common and best characterized epigenetic abnormality in human malignancies is DNA hypermethylation. Previous studies have reported polycomb group (PcG) target genes to be more frequent targets of aberrant silencing by DNA methylation than non-PcG target genes.[11-13] These genes are targets for PcG complexes, which are the determining factor in cell lineage choices during embryogenesis. These proteins are needed to maintain the correct identities of stem cells, progenitor cells and differentiated cells. According to current hypotheses, deregulated repression of PcG target genes results in the accumulation of a population of cells which are not able to respond to differentiation signals, leading to loss of cell identity and ultimately to cancer.

Since it has been shown that this aberrant methylation of PcG target genes plays an important role in the formation of many carcinomas,[14] we hypothesized that PcG target gene hypermethylation might be an early event in bladder carcinogenesis and that these epigenetic aberrations might therefore also be observed in tumors of young patients. In order to define epigenetic characteristics more specifically, we divided patients into four different age categories i.e. <20 years, 20-40 years, 40-60 years and >60 years.

MATERIAL AND METHODS

Patient population and tissue collection

Patients 167 patients were included in this study and were divided according to age into four different age categories, i.e. <20 years (n=14), 20-40 years (n=52), 40-60 years (n=47) and >60 years (n=58). Patients <20yr, treated between 1991 and 2009 for UCC, were retrieved from the PALGA-Database (The nationwide registry of histo- and cytopathology in the Netherlands). Tissue blocks from patients 20-40yr were collected from the pathological archive of the Leiden University Medical Centre, Leiden and from our own archive. Tumors from patients above the age of 40 were randomly included from our own pathological archive. These patients were all treated between 1990 and 2012. Urines from 35 healthy controls >50yr were retrieved from a previous study[15] and served as the equivalent of normal urothelium. Tissue for DNA extraction was obtained by manual dissection from formalin-fixed, paraffin-embedded (FFPE) blocks, containing tumor areas that were selected by pathological examination of the corresponding histological slides. Tissue slides were deparaffinised with xylene and ethanol and DNA was isolated using the DNeasy Tissue kit (Qiagen, Hilden, Germany), according to manufacturers' protocol.

Gene panel selection

Five PcG target genes were selected from the findings of a genome-wide methylation study in bladder cancer.[16] Genes were selected based on two selection criteria. Firstly, genes with the highest average delta beta and beta ratio were selected. The beta-value could be interpreted as the percentage methylation at a certain CpG-site. The selected genes had the highest discrepancy in methylation between cancer and urine from healthy controls. Secondly, the genes were down-regulated in bladder cancer according to the Oncomine Database™.

Methylation analysis

Methylation analysis was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, California, USA) according to the manufacturer's protocol. Briefly, DNA was treated with sodium bisulfite, followed by bisulfite-specific PCR for the five regions of interest. For each PCR reaction a DNA input of 20ng and a PCR primer concentration of 20 pM was required. After PCR, a Single Nucleotide Primer Extension (SNuPE) analysis was performed, using primers that annealed to the PCR product adjacent to the cytosine of interest. SNuPE probes were extended with a labelled dideoxynucleotide and the products were analyzed on an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a methylated cytosine. For each gene, the methylation ratio was calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peaks**.** Primer and probe concentrations are given in table 8.1.

Table 8.1: SNuPE primers and probes

BSP primers						
MEIS1	GGGTTTTTAGAGGTTAGGGGAA					
	CAACTAAATAACCAAACCTCTCCTC					
ONECUT2	GGGGTTTTTTGTTTTTTGTATTTTT					
	TCATTTTCAAACTTAAACTTAATCACC					
OTX1	TTTTGAGAGGTATAGAGAGGGGTAGT					
	CCCCTAACAAACCCAAATCTC					
PCDH7	TGATTGTTAAGGTGGGAGATATTAA					
	TATTAACCAAAATATCCCCAAAATC					
SOX21	GGTTATTTAGTGTGTGTATATGAGAG					
	AAAACCCTTATTTTCTAAAATCTAC					
SNuPE probes	**Sequence (5′ > 3′)**	**Size (bp)**	**Strand**	**UM**	**M**	**µM**
MEIS1	T44 GGAGAGGGGGGTTATGATGTTAGG	68	Sense	T	C	0.2
ONDECUT2	T58 GTTTGGGYGGTTGGGTT	75	Sense	T	C	0.2
OTX1	T35 ATTTGTGGTTTTTTAGGTT	54	Sense	T	C	0.2
PCDH7	T18 CCCCATCACAAAAAAATCCAAC	40	Antisense	A	G	0.2
SOX21	T9 ATATTTAGTTTTGATATTTTT	30	Sense	T	C	0.2

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences 17.0 (SPSS Statistics 17.0). The Kruskal-Wallis Test was used to determine the difference in methylation ratios between the age groups. Results were considered statistically significant if $p<0.05$.

RESULTS

Patient and tumor characteristics

A total of 167 patients with a primary bladder tumor were included in this study. Patients were divided into four different age groups; 14 patients in the group <20yr, 48 patients in the group 20-40yr, 47 in the 40-60yr patient group, and 58 patients in the age group >60yr. Patient and tumor characteristics are depicted in table 8.2. 130/167 (78%) patients were male. Male:female ratio was 3:1 in the highest three age groups. In the youngest age group more males were affected than females, with a male:female ratio of 13:1. Tumors in all age groups were predominantly of low stage (pTa; 65%) and grade (G1-2; 78%). Tumors in the youngest age group were of significantly lower stage and grade compared to the >60yr group ($p<0.05$).

Gene panel selection

Based on the selection criteria described above (see materials and method), five PcG target genes were selected i.e. *MEIS1, ONECUT2, OTX1, PCDH7* and *SOX21*. Beta values of cancer

Table 8.2: Patient and tumor characteristics (n=167)

	<20 yr	20-40 yr	40-60 yr	>60 yr	Total
	n (%)	n (%)	n (%)	n (%)	n (%)
Gender					
Male	13 (93)	37 (77)	37 (79)	44 (76)	131 (78)
Female	1 (7)	11 (23)	10 (21)	14 (24)	36 (22)
Stage					
pTa	13 (93)	32 (67)	32 (68)	31 (53)	108 (65)
pT1	1 (7)	7 (14)	9 (19)	18 (31)	35 (21)
≥pT2	-	8 (17)	5 (11)	8 (14)	21 (12.5)
pTis	-	1 (2)	-	-	1 (0.5)
pTx	-	-	1 (2)	1 (2)	2 (1)
Grade					
G1	10 (71)	21 (44)	20 (43)	14(24)	65 (39)
G2	4 (29)	13 (27)	18 (38)	30 (52)	65 (39)
G3	-	13 (27)	7 (15)	13 (22)	33 (20)
Gx	-	1 (2)	2 (4)	1 (2)	4 (2)

DNA in relation to beta values of urine DNA from healthy controls were derived from data from a genome-wide methylation study[15] and are depicted in Figure 8.1. The selected PcG target genes had the highest beta-ratio and delta-beta values; these genes had the highest discrepancy in methylation between cancer DNA and urine DNA from healthy controls (Figure 8.1).

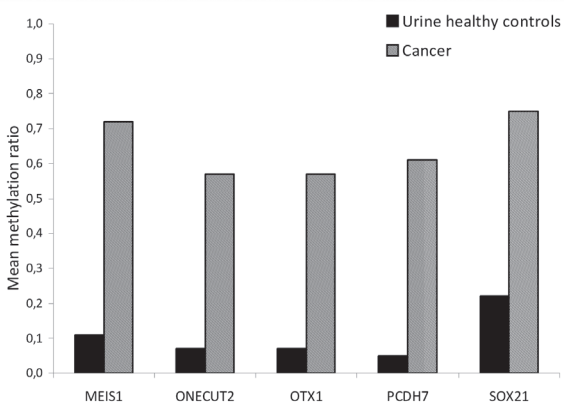

Figure 8.1 *Methylation ratios per gene in bladder cancer compared to urine from healthy controls. Data is derived from a genome wide methylation study (Kandimalla et al, Eur Urol, 2011)*

Methylation analysis

Methylation analysis was performed for each selected gene. Since we were looking for an initiating event in bladder carcinogenesis, genes with equal methylation ratios in all four age groups were of interest. *ONCECUT2, OTX1* and *SOX21* showed a significant difference in methylation ratios between the four age groups; *ONECUT2* (p<0,001), *OTX1* (p<0,001) and *SOX21* (p<0,001). As shown in figure 8.2A and 8.2B *ONECUT2* and *OTX1* had significantly less methylation in the <20yr group compared to the three older age groups. *SOX21* (figure 8.2C) had significantly higher methylation ratios in the >60yr age group compared to the three younger age groups. MEIS1 and *PCDH7* showed similar methylation ratios in all four age groups, p-value 0.17 and 0.10 respectively. However the median methylation ratios for *MEIS1* were low in all age groups (<20yr: 0.06, 20-40yr: 0.24, 40-60yr: 0.06 and >60yr: 0.18, figure 8.2D). *PCDH7* showed high median methylation ratios in all four age groups as depicted in figure 8.3. Although, the median methylation ratio did increase with age, i.e. <20yr: 0.44, 20-40yr: 0.60, 40-60yr: 0.61 and >60yr: 0.71. We therefore decided to perform additional methylation analysis for *PCDH7* in 35 urine samples from healthy controls (age >50yr). The amount of methylation in the healthy controls was significantly less than that in the four patient groups (median 0.22; p<0.001).

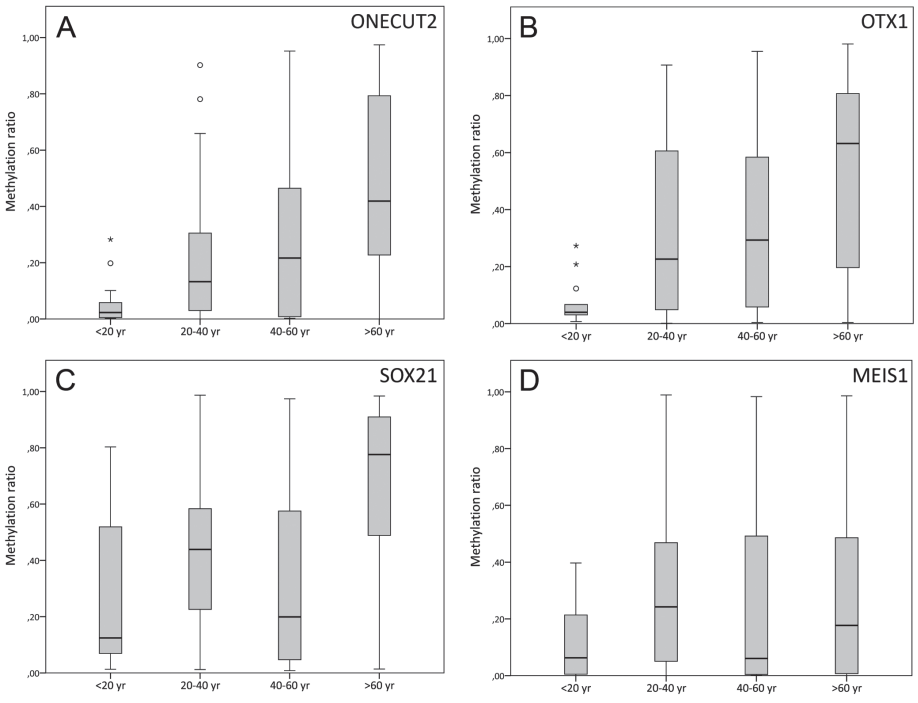

Figure 8.2 *Methylation ratios by patient age for: (A) ONECUT2 (P<0,001), (B) OTX1 (P<0,001), (C) SOX21 (P<0,001) and (D) MEIS1 (P=0,17). Boxplots indicate 25% to 75% quartile range. Horizontal lines indicate median.*

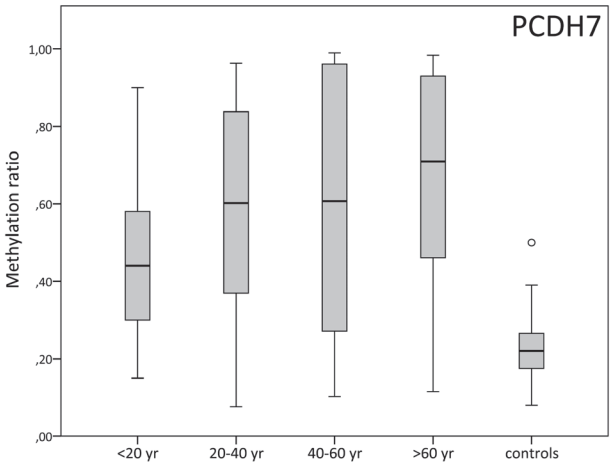

Figure 8.3 Methylation ratios by patients age for PCDH7. Methylation ratios were not significantly different in the four age groups (p=0,10) Median of age groups are 0,44; 0,60; 0,61 and 0,71, respectively. There was significantly less methylation in the normal controls compared to the patient groups (median 0,22; p<0.001). Boxplots indicate 25% to 75% quartile range. Horizontal lines indicate median.

DISCUSSION

Previous findings suggest that BC in patients <20 years comprise a biologically distinct group. These tumors seem to be genetically stable, they lack the typical genetic aberrations found in older patients and are often of low stage and low grade.[9, 17] In this study we aimed to elucidate bladder carcinogenesis in young patients by investigating the methylation status of five PcG target genes. We divided the included patients into four different age categories, i.e. <20yr, 20-40yr, 40-60yr and >60yr. Overall, tumors from young patients had substantially less methylation for most of the markers compared to tumors from patients in the older age groups. Only PCDH7 showed high median methylation ratios in all four age groups.

To our knowledge, hypermethylation in patients <20yr has only been investigated in one previous study.[10] Owen et al. studied the methylation status of eight genes that were already known to be associated with bladder carcinogenesis, i.e. *TNRFSF25, EDNRB, WIF1, APC, BCL2, MGMT, Cyclin D2* and *E-Cadherin*. They analyzed tumors from 12 patients under the age of 20 and compared the methylation results with those of tumors in patients aged 20-45 years and in patients aged above 45 years. Most markers had significantly less methylation in the youngest group. Only *TNFRSF25* and *cyclin D2* showed similar methylation rates in all age groups. However, overall methylation rates for *cyclin* D2 were low in all three age groups. Methylation rates for *TNFRSF25* were high, suggesting a role in early development of bladder tumors. Yet, the authors did not include negative controls in their study. In a previous study,[18] we found high methylation of *TNFRSF25* in urine DNA from healthy controls, raising the hypothesis that *TNFRSF25* does not play an important role in urothelial tumorigenesis. Furthermore, since

T*NFRSF25* and *cyclin* D2 are not regulated by PcG target genes, it was beyond the scope of our study to investigate their methylation rates.

We found *PCDH7* with similar methylation ratios in all age groups. We therefore suggest that *PCDH7* is an interesting candidate in early bladder tumorigenesis. *PCDH7*, also known as *BH-PCDH*, is a gene located on the p-arm of chromosome 4 and is a member of the protocadherin family, which is a subgroup of the cadherin superfamily. Protocadherins are predominantly expressed in the brain and they function as cell-cell recognition molecules.[19, 20] There is less known about the function of the *PCDH7* gene in bladder cancer. In a gene-profiling study Sanchez-Carbayo et al found significant downregulation of *PCDH7* in bladder cancer by investigating tissue from 52 normal urothelium and 105 bladder tumors.[21] Similarly, Djyrskøt et al analyzed 60 bladder tumors and also found *PCDH7* underexpression.[22] In our study we did not investigate *PCDH7* gene expression, but only studied DNA-hypermethylation. However aberrant DNA-hypermethylation often results in gene silencing and this should be confirmed by functional studies.

The major limitations in this study were the small number of tumor samples from patients in the youngest age group and the fact that these samples were collected from the national pathological database. Since the database only provides anonymous pathological information, we were not able to complete follow-up of these patients with regard to disease recurrence and progression. Neither are we able to follow these patients over time.

CONCLUSIONS

Bladder cancer patients aged <20yr seem to form a clinically and molecularly distinct group. Epigenetically, most markers showed lower methylation rates in patients <20yr. Only *PCDH7* showed similar high methylation ratios in all age groups and therefore might play a role in early urothelial carcinogenesis. Future studies with larger patient populations may provide more insight into the role of epigenetic silencing of Polycomb target genes in tumorigenesis.

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Chapter 9

HRAS mutations in bladder cancer at an early age and the possible association with the Costello Syndrome

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ABSTRACT

Bladder tumors of patients <20yr have a low incidence of genetic aberrations typically found in tumors in older patients. In this study, we investigated oncogene mutations in patients with bladder cancer <20yr and compared them to older age groups. Interestingly, we observed a relatively high number of *HRAS* mutations in tumor from young patients. These mutations were also highly uncommon in bladder cancers of older patients, i.e. p.(Gly12Ser) and p.(Gly12Ala). Germline mutations in the *HRAS* gene, especially p.(Gly12Ser/Ala) cause Costello Syndrome (CS), a severe congenital disorder. Indeed, one of the patients had been diagnosed with CS. We hypothesized that some of the other patients might be mosaic for the *HRAS* mutation and therefore could express some of the clinical features of CS, like tumor predisposition. Hence we isolated DNA from microdissected stroma and analyzed it for *HRAS* mutations. In the CS patient and in patient X the mutation was also highly expressed in normal stroma. We conclude that patient X is possibly mosaic for the *HRAS* mutation. These results suggest that mosaicism for oncogenic *HRAS* mutations may increase the risk for developing bladder cancer at a young age.

INTRODUCTION

Bladder cancer (BC) is a disease of the elderly with a peak incidence in the sixth decade of life and only 1-2.4% of all cases present under the age of 40. BC in patients younger than 20 years is even more uncommon with reported incidence rates of only 0.1-0.4%.[1] Previous studies demonstrated that tumors of patients <20 years lack or have a much lower incidence of epigenetic and genetic aberrations typical of bladder cancer in elderly patients.[2]

Together with mutations in *FGFR3* and *PIK3CA*, mutations in the *RAS* genes are the most common mutations found in BC and up to 13% of all bladder tumors harbour a mutation in *HRAS*, *KRAS* or *NRAS*.[3] There is limited data available regarding these oncogenic mutations in BC of patients <20 years. Therefore, we aimed to investigate *FGFR3*, *PIK3CA* and *RAS* mutations in BC of patients <20 years and compared the results with the mutation status of a control group consisting of patients with BC >20 year.

MATERIAL AND METHODS

Fourteen patients <20 years (median 16.5; range 11-19) were included. In addition, three different control groups were investigated, i.e. patients diagnosed with BC at 20-40 years (n=43), 40-60 years (n=45), and >60 years (n=58). Tumor tissue was obtained from a previous study.[41] Normal surrounding stroma was microdissected ensuring >90% normal cells. Samples were used in accordance with the Dutch Code for "Proper secondary use of human tissues" (http://www.federa.org). Tumor DNA was analyzed for mutations in *HRAS*, *KRAS* and *NRAS* with a multiplex Snapshot assay as described previously.[5] This assay identifies 96% of the *RAS* mutations in BC, according to the Sanger institute. In addition, the DNA was screened using the Snapshot analysis for mutations in *PIK3CA* and *FGFR3*.[5] Reference sequences are shown in supplementary file 9.1. Sequences and conditions of primers and probes are depicted in supplementary table 9.1 and 9.2.

RESULTS

Table 9.1 shows the mutations found in the different age groups. FGFR3 and PIK3Ca mutations are more abundant in tumors from patients over 40 years of age. Overall, the *FGFR3* mutation co-occurred with a mutation in *PIK3CA* in 24 cases, similar to previous observations. A mutation in the *FGFR3* gene together with a mutation in *KRAS* was found in only one patient. In the youngest age group, no mutations were found in *PIK3CA* and only one mutation was observed in the *FGFR3* gene. However, 4/14 (29%) of the young patients harboured a mutation in *HRAS,* a much higher percentage than in the older age groups (Table 9.1). Interestingly, not only did we observe this high number of HRAS mutations, but also 3/4 of these mutations were highly

Table 9.1: Distribution of oncogene mutations in bladder tumours of different age categories

		Age categories			
		<20yr	20-40yr	40-60yr	>60yr
Mutation		n=14	n=43	n=45	n=58
HRAS	p.(Gly12Ala)	1			
	p.(Gly12Ser)	2	1		
	p.(Gly12Cys)			1	
	p.(Gly13Arg)				1
	p.(Gln61Leu)		2		1
	p.(Gln61Arg)	1	1	1	1
KRAS	p.(Gly12Cys)		1	2	1
	p.(Gly12Asp)		1	1	1
	p.(Gly12Val)		1	1	1
Wild type		10	36	39	52
FGFR3	p.(Arg248Cys)		1	2	4
	p.(Gly372Cys)			4	1
	p.(Ser249Cys)	1	12	18	26
	p.(Tyr375Cys)			8	5
	p.(Arg248Cys)+ p.(Ser249Cys)				1
Wild type		13	29	13	21
Unknown			1		
PIK3CA	p.(Glu542Lys)		2	2	3
	p.(Glu545Gly)			1	1
	p.(Glu545Lys)		1	6	8
	p.(His1047Arg)			2	2
	p.(His1047Leu)				2
Wild type		13	40	34	42
Unknown		1			
Co-occurrence of mutations					
FGFR3 and *PIK3CA* mutation			2	8	14
FGFR3 and *KRAS* mutation				1	
KRAS and *PIK3CA* mutation			1	1	
HRAS and PIK3CA mutation					1
FGFR3, K*RAS* and *PIK3CA* mutation				1	1

uncommon in BC, i.e. c.34G>A (p.(Gly12Ser)) and c.35C>G (p.(Gly12Ala)). In addition, another p.(Gly12Ala) mutation was found in a bladder tumor of a 29yr-old patient from the control group. This patient was a known CS patient. The four patients with the uncommon p.(Gly12Ser/Ala) mutation all had a solitary TaG1 bladder tumor. In these four patients *RAS* mutation analysis was performed on DNA isolated from microdissected tumor surrounding stroma. The areas of stroma used as normal surrounding tissue are depicted in Figure 9.1a. For both tumor and

normal tissue, the results of the *RAS* mutation analysis are shown in Figure 9.1b. The stroma of patient #1, a 16yr-old boy with a p.(Gly12Ala) *HRAS* mutation in his bladder tumor, did also display this *HRAS* mutation. Likewise, although the signal was low, a p.(Gly12Ser) *HRAS* mutation was found in the normal stroma of patient #2, an 18yr-old boy with a p.(Gly12Ser) *HRAS* mutation in his bladder tumor. There was no mutation found in the normal tissue of patient #3, a 16yr-old male. In the 29yr-old female CS patient (Patient #4) from the control group the p.(Gly12Ser) *HRAS* mutation was also found in the surrounding stroma.

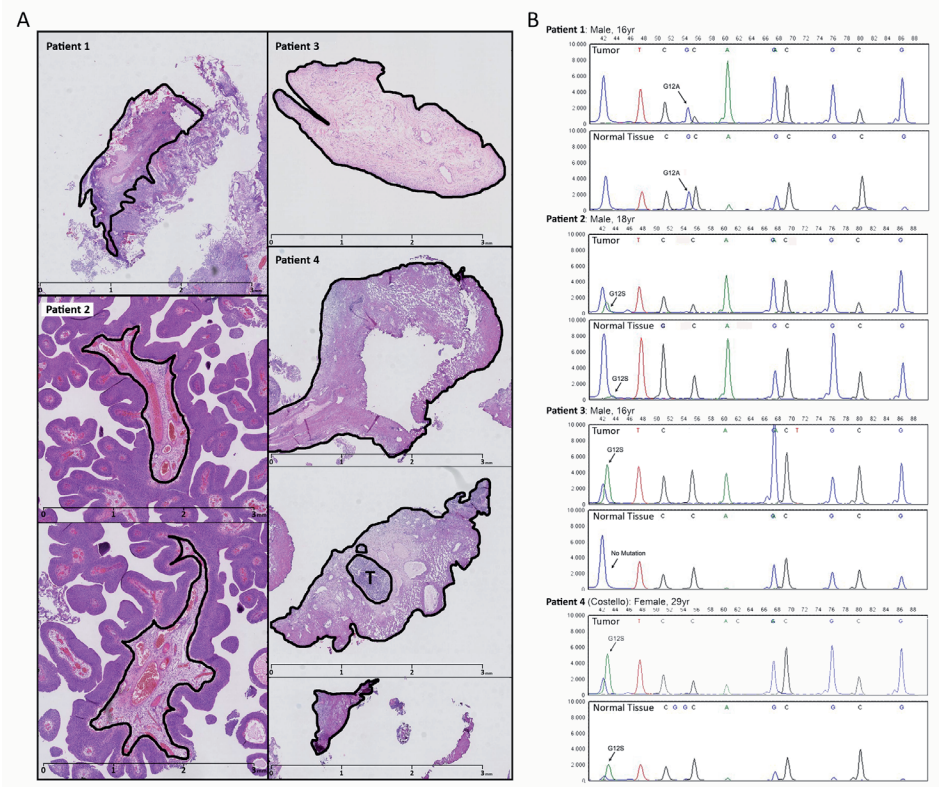

Figure 9.1 *(A) Microdissected areas used as normal surrounding tissue in the SNaPshot analysis. Microdissection ensured >90% normal stromal cells. Stroma from patient #1, 2 and 4 was derived from the same side as the bladder tumour. Stroma from patient #3 was obtained from random bladder biopsies. (B) RAS snapshot analysis of four patients' tumour tissue and normal surrounding stroma. T = Tumour tissue*

DISCUSSION

BC of patients <20 years seem to form a molecular distinct group compared to BC of the elderly. In this study we screened BC of patients <20 years for oncogenic mutations that are typically for found in BC of the elderly. As expected, no mutations were found in the *PIK3CA* gene, and only

one mutation in the *FGFR3* gene. However a high amount of uncommon p.(Gly12Ser/Ala) *HRAS* mutations were observed in the youngest age category.

Germline mutations in the *HRAS* gene are found in several developmental disorders, such as the Costello Syndrome (CS). CS is a rare syndrome with only 300-400 reported cases worldwide. CS is caused by point mutations in the *HRAS* gene, mostly resulting in p.(Gly12Ser) or p.(Gly12Ala). This multisystem disorder causes mental retardation, distinctive facial appearance, cardiovascular abnormalities, skin and musculoskeletal abnormalities, and tumor predisposition. Most frequent malignancy found is the embryonal rhabdomyosarcoma, but also cases with BC have been described.[6] It appeared that the 29 yr-old patient with a p.(Gly12Ser) *HRAS* mutation in the bladder tumor was known to have CS and therefore the p.(Gly12Ser) *HRAS* mutation could also be observed in the normal surrounding stroma. The mutation found in the normal tissue of patient #2 could be due to contamination of tumor cells, since the signal is mutant peak is low compared to the wild-type peak. However, the mutation signal in the normal tissue of patient #1 is too high to be caused by contamination of tumor cells during microdissection. This suggests that this patient could be mosaic for the p.(Gly12Ala) *HRAS* mutation and therefore might have some of the clinical features of CS, such as tumor predisposition. Blood or other patients' tissue of would be necessary to strengthen this hypothesis. Unfortunately, due to ethical limitations in accessing clinical data, we were unable to obtain additional tissue or information on possible other CS-like features of patients #1 (and patient #2).

Mosaicism of *HRAS* mutations in relation with CS was described previously in a small number of patients.[7, 8] The phenotype of patients with mosaicism may differ and the clinical manifestations could occur along a spectrum depending on the affected tissue compartments.[9] In a recently published case report, mutant *HRAS* mosaicism was also described in relation to BC. The publication described a patient with an extensive mosaicism for a *HRAS* p.(Gly12Ser) mutation. This 49yr-old man presented with widespread congenital epidermal nevi, multiple bladder tumors and a BC lung metastasis. The *HRAS* p.(Gly12Ser) mutation was found in the epidermal nevi as well as in the multiple bladder tumors and lung metastasis.[10] The authors concluded that these findings corroborate the theory that *RAS* mutations may occur during early embryogenesis and could contribute to carcinogenesis at later age. Our results contribute to this hypothesis and suggest that mosaicism for oncogenic *HRAS* mutations may even increase the risk for developing bladder cancer at very young age. Thus, *RAS* mutation analysis should be performed on bladder tumors of young patients and if positive, the patient should be screened for possible manifestations of CS.

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SUPPLEMENTARY FILES

Supplementary table 9.1: Primer sequences mutation analysis

FGFR3 PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
FGFR3 RI Fw	AGTGGCGGTGGTGGTGAGGGAG	115	1.2
FGFR3 RI Rev	GCACCGCCGTCTGGTTGG		1.2
FGFR3 RII Fw	CAACGCCCATGTCTTTGCAG	138	0.7
FGFR3 RII Rev	AGGCGGCAGAGCGTCACAG		0.7
FGFR3 RIII Fw	GACCGAGGACAACGTGATG	160	0.7
FGFR3 RIII Rev	GTGTGGGAAGGCGGTGTTG		0.7
Pan-RAS PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
HRAS exon1 Fw	CAGGAGACCCTGTAGGAGG	139	0.6
HRAS exon1 Rev	TCGTCCACAAAATGGTTCTG		0.6
HRAS exon2 Fw	GGAGACGTGCCTGTTGGA	140	0.3
HRAS exon2 Rev	GGTGGATGTCCTCAAAAGAC		0.3
KRAS exon1 Fw	GGCCTGCTGAAAATGACTG	163	0.3
KRAS exon1 Rev	GGTCCTGCACCAGTAATATG		0.3
KRAS exon2 Fw	CCAGACTGTGTTTCTCCCTT	155	0.3
KRAS exon2 Rev	CACAAAGAAAGCCCTCCCCA		0.3
NRAS exon1 Fw	GGTGTGAAATGACTGAGTAC	128	0.3
NRAS exon1 Rev	GGGCCTCACCTCTATGGTG		0.3
NRAS exon2 Fw	GGTGAAACCTGTTTGTTGGA	103	0.3
NRAS exon2 Rev	ATACACAGAGGAAGCCTTCG		0.3
PIK3CA/NRAS PCR Primermix			
Primer	Sequence (5'->3')	Product size (bp)	Concentration in PCR mix (µM)
PIK3CA ex9-Fw	AGTAACAGACTAGCTAGAGA	139	1
PIK3CA ex9-Rev	ATTTTAGCACTTACCTGTGAC		1
PIK3CA ex20-Fw	GACCCTAGCCTTAGATAAAAC	109	0.7
PIK3CA ex20-Rev	GTGGAAGATCCAATCCATTT		0.7
NRAS ex.2 Fw	GGTGTGAAATGACTGAGTAC	128	0.3
NRAS ex.2 Rev	GGGCCTCACCTCTATGGTG		0.3
NRAS ex.3 Fw	GGTGAAACCTGTTTGTTGGA	103	0.5
NRAS ex.3 Rev	ATACACAGAGGAAGCCTTCG		0.5

Supplementary table 9.2: Probe sequences mutation analysis

FGFR3 Probe mix						
Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
S373C	T19 GAGGATGCCTGCATACACAC	39	sense	T	A	0.4
K652M/T	T20 CACAACCTCGACTACTACAAGA	42	sense	A	T/C	0.7
G372C	T29 GGTGGAGGCTGACGAGGCG	48	sense	G	T	0.2
A393E	T34 CCTGTTCATCCTGGTGGTGG	54	sense	C	A	0.7
R248C	T46 CGTCATCTGCCCCCACAGAG	66	sense	C	T	0.4
Y375C	T43 ACGAGGCGGGCAGTGTGT	61	sense	A	G	0.6
S249C	T36TCTGCCCCCACAGAGCGCT	55	sense	C	G	0.8
K652Q/E	T50 GCACAACCTCGACTACTACAAG	72	antisense	A	C/G	0.3
G382R	T56 GAACAGGAAGAAGCCCACACC	76	antisense	C	T	0.4
Ras Set 1 Probe mix						
Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
HRAS pos.34	T17 CTGGTGGTGGTGGGCGCC	35	sense	G	C/T/A	0.5
HRAS pos.182	T18 GCATGGCGCTGTACTCCTCC	38	antisense	T	G/C/A	0.15
KRAS pos.34	T25 GGCACTCTTGCCTACGCCAC	45	antisense	C	G/A/T	0.5
HRAS pos.35	T31 CGCACTCTTGCCCACACCG	50	antisense	C	G/A/T	0.7
NRAS pos.182	T33 GACATACTGGATACAGCTGGAC	55	sense	A	G/C/T	0.5
KRAS pos.181	T41 CTCATTGCACTGTACTCCTCTT	63	antisense	G	T/C	0.2
HRAS pos.181	T46 CATCCTGGATACCGCCGGC	65	sense	C	A/G	0.7
KRAS pos.35	T49 AACTTGTGGTAGTTGGAGCTG	70	sense	G	C/T/A	0.2
HRAS pos 37	T55 CAGCGCACTCTTGCCCACAC	75	antisense	C	G/A/T	0.7
NRAS pos.34	T62 CTGGTGGTGGTTGGAGCA	80	sense	G	C/T/A	0.2
PIK3CA/NRAS Probe mix						
Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT	µM*
E542K	T17 ACACGAGATCCTCTCTCT	35	sense	G	A	0.15
E545G	T21 CCTCTCTCTGAAATCACTG	40	sense	A	G	0.5
E545K	T25 ATCCTCTCTCTGAAATCACT	45	sense	G	A	0.3
H1047R	T30 GAAACAAATGAATGATGCAC	50	sense	A	G	0.3
NRAS pos.34	T34 GTGCGCTTTTCCCAACACCAC	55	antisense	C	G/A/T	0.5
NRAS pos.35	T41 CTGGTGGTGGTTGGAGCAG	60	sense	G	C/A/T	0.3
NRAS pos.37	T46 GGTGGTGGTTGGAGCAGGT	65	sense	G	C/A/T	0.1
NRAS pos.38	T49 GTCAGTGCGCTTTTCCCAACA	70	antisense	C	G/A/T	0.5
NRAS pos.180	T54 GGACATACTGGATACAGCTGG	75	sense	A	T	0.3
NRAS pos.181	T58 CTCATGGCACTGTACTCTTCTT	80	antisense	G	C/T	0.2
NRAS pos.182	T63 GACATACTGGATACAGCTGGAC	85	sense	A	C/G/T	0.2
NRAS pos.183	T68 CTCTCATGGCACTGTACTCTTC	90	antisense	T	A/G/C	0.7

* Concentration in reaction

PART V

GENERAL DISCUSSION AND SUMMARY/
SAMENVATTING

Chapter 10

General discussion

GENERAL DISCUSSION

Bladder cancer is the fifth most common cancer in the western world.[1] It is a disease of the elderly with a peak incidence in the 6th decade of life. Approximately 80% of the new cases will present as non-muscle invasive disease with an overall favorable prognosis. Yet, these tumors tend to recur with a 5-year recurrence rate varying between 31-78%.[2] Furthermore, there is a chance of progression to muscle invasive disease with a much worse prognosis. The high recurrence rate and risk of progression necessitates life-long frequent follow-up by cystoscopy as recommended by the EAU guidelines.[3, 4] The high recurrence rate together with overall good survival result in high costs and patient burden. Development of non-invasive tests and risk stratification at first diagnosis will contribute to reduction of costs and improvement of patient burden.

Part II of this thesis investigates molecular markers in relation to disease progression. It includes the validation of progression markers in patients with primary low-grade disease. Based on the results we defined new molecular grades in order to divide patients in different risk groups and determined whether these risk groups could predict patient outcome. Furthermore, the prevalence of *TERT* mutations in BC and its relation to patient outcome was studied. In **part III** we investigated the role of urinary markers in the follow-up of non-invasive bladder cancer patients. In addition, markers were tested on patients presenting with hematuria to study the value of urinary makers at initial diagnosis. **Part IV** focuses on the genetic and epigenetic alterations in patients with bladder cancer at an early age in order to get a better insight in early bladder carcinogenesis.

New molecular markers and their relation to disease progression

Ta–tumors form the largest group of bladder cancer, comprising approximately 70% of all NMIBC.[5] Although recurrence rates are high, low-grade (G1/G2) Ta-tumors have an overall good prognosis with limited progression rates varying between 1-6%, based on the EORTC risk-scores developed by Sylvester *et al*.[2] This risk calculation is highly dependent on pathological review. Previous research demonstrated that the pathological assessment is subject to high interobserver and intraobserver variability, resulting in poor reproducibility. Molecular markers could help to objectify tumor aggressiveness and improve the accuracy of risk-prediction. This could result in more adjusted follow-up schemes and decrease the number of cystoscopies in low-risk patients. Up to now, no molecular markers are implemented in the risk calculator included in the EAU guidelines. Kandimalla *et al* identified *GATA2*, *TBX2*, *TBX3*, and *ZIC4* as progression markers for primary low-risk disease in a genome wide methylation study.[6] In this study DNA of fresh frozen tissue was used. In **Chapter 2** we validated these four methylation makers in a large set of Formalin-Fixed Paraffin-Embedded tissue from primary low-grade NMIBC. A total of 192 primary tumors from patients with grade 1 or 2 NMIBC were included of who 77 progressed to muscle invasive disease. Methylation analysis was done for all four markers by using the MS-SNaPshot:

an easy to perform test, which is highly reproducible. Cox-regression analysis was performed and showed the independent effect of *TBX2*, *TBX3* and *ZIC4* on progression-risk, with *TBX2* and *TBX3* as best predictors.

TBX2 and TBX3 play a critical role in embryonic development and deregulation of these two genes in relation to tumor progression is described in several cancer types. TBX2 and TBX3 are both interfering with the p53-pathway by repressing p14/ARF and p21/CIP1. *P53* mutations are frequently found in aggressive bladder tumors. Inhibiting this pathway results in uncontrolled proliferation and cell survival. Methylation of TBX2/3 could be an alternative way of deactivating the p53 pathway.[7, 8] Reinert *et al* also investigated methylation in relation to disease progression in pTa tumors and found that *TBX4* was more frequently hypermethylated in progressive tumors compared to the non-progressive pTa-tumors.[9] These findings in combination with our own results imply the role of T-Box genes in tumor progression.

Based on the methylation status of *TBX2* and *TBX3* we determined new molecular grades. High molecular grade (HmG) was defined as high methylation ratios for both *TBX2* and *TBX3*. Hypermethylation of only *TBX2* or *TBX3* was labeled as intermediate molecular grade (ImG). Low molecular grade (LmG) included patients with low methylation rates for both *TBX2* and *TBX3*. Progression-free survival was significantly better for patients in the LmG-group compared to patients with a tumor of ImG or HmG. Likewise, patients with ImG showed better progression-free survival compared to the HmG-group. Previously, molecular grading was introduced by van Rhijn *et al* based on MIB-1 expression and *FGFR3* mutation status.[10] In that study, molecular grade 1 was defined as low expression of MIB-1 and *FGFR3* mutant, molecular grade 2 included tumors with low expression of MIB-1 and *FGFR3* wild-type or high expression of MIB-1 and *FGFR3* mutant. Molecular grade 3 comprised tumors with high expression of MIB-1 and *FGFR3* wild-type. The combination of MIB-1 and *FGFR3* mutation status was a significant predictor of progression and was superior to clinical tumor characteristics. In a next study, van Rhijn *et al* combined the molecular grade with the EORTC risk scores and showed increased accuracy of progression prediction based on the EORTC risk protocols in combination with molecular grade, compared to the EORTC risk protocol alone.[11] We presume that our methylation markers could further improve the predictive accuracy. Still, these studies include relatively small retrospective patients series. Large prospective studies should investigate the additional value of these molecular markers in combination the EORTC risk protocol in order to justify adjustment of the current risk stratification.

In **chapter 3** the presence of *TERT* mutations in BC was examined in relation to patient outcome. We found a mutation frequency of 70% in 111 fresh frozen tumor samples of all stages, with C228T and C250T as most frequently observed mutations. Next, FFPE tissue from an independent set of 357 BC patients was investigated with the SNaPshot analysis including the two most frequent mutations and showed a percentage of *TERT* mutations of 79%. *TERT* mutation status was not associated with patient age, sex or smoking status. Heterogeneity was assessed by analyzing multiple areas within a tumor and revealed an overall consistency, suggesting the early appearance of TERT mutations in bladder carcinogenesis. There was no relation

between the *TERT* mutation status and progression-free survival, disease-specific survival and overall survival. Urine analysis with the *TERT* SNaPshot assay showed a sensitivity for recurrence detection of 42%, which was superior to the *FGFR3* analysis, with a sensitivity of 19%. Likewise, 62% of the primary tumors were detected by the *TERT* assay, compared to 36% with the *FGFR3* assay. The *TERT* and *FGFR3* analysis combined identified 50% of all recurrent tumors and 70% of the initial BCs. Kinde *et al* also investigated the presence of *TERT* mutations in BC and found similar results with a reported mutation frequency of 74%, predominantly C228T and C250T. No relation with disease progression was found. Additionally, no mutations were observed in the normal surrounding urothelium. Moreover, urine samples from patients with a *TERT* mutant tumor were investigated and 7/7 of the patients with a positive urine sample developed a recurrence within 3.5 months.[12] In the same line, Hurst *et al* found a TERT mutation frequency of 83% in BC independent from stage or grade and a sensitivity of 79% for the detection of BC in urine. Yet, they did not distinguish between primary and recurrent disease.[13] The presence of *TERT* mutation in both NMIBC and MIBC suggests the involvement of *TERT* in both pathways. The high frequency of *TERT* mutations in combination with the high sensitivity for tumor detection in urine makes the *TERT* mutation assay a promising tool for the diagnosis and follow-up of BC.

The use of molecular markers in the follow-up of non-muscle invasive bladder cancer

There is a need for highly sensitive and specific markers in order to safely reduce the number of cystoscopies during surveillance of patients with non-muscle invasive bladder cancer (NMIBC). In search for novel markers, it is important to include the correct urine samples in order to avoid unreliable results. Most studies include a mixture of primary and recurrent tumors. Primary tumors are often larger in size and less well-differentiated then their recurrent counterparts. Therefore, these tumors will shed more tumor cells compared to recurrent tumors, resulting in misleading high marker performance.[14] For the development of urinary assays, which are intended for surveillance purposes, we advocate the use of urine samples collected during follow-up. In the same line, it is important to distinguish between patients with primary high-grade or low-grade disease for the calculation of marker performance. Patients with primary low-grade disease are most suitable for follow-up by urine analysis, yet their low-grade recurrences are mostly harder to detect. In all studies included in this thesis, we clearly distinguished between urines collected during follow-up or collected at first visit.

In **chapter** 4, we designed a bladder cancer specific MS-MLPA (BC MS-MLPA) for the detection of recurrent NMIBC. Multiple studies have shown the relation of promoter hypermethylation and bladder carcinogenesis, and several studies reported the use of methylation markers in order to detect bladder cancer in voided urine.[15] The MS-MLPA analysis is based on a multiplex PCR to investigate multiple CpGs in one assay and requires only 50ng DNA. The BC MS-MLPA was constructed based on the commercially available MS-MLPA kits -001 and -002. In addition, genes were selected from the literature, that were known to be frequently methylated in blad-

der cancer. The BC MS-MLPA included 23 probes for 12 different genes. We finally selected a four gene panel consisting of *APC_a*, *TERT_a*, *TERT_b* and *EDNRB*, which resulted in a sensitivity of 63% and specificity of 58% for recurrence detection in the test set, consisting of 49 pre-TUR urine samples and 60 urine samples from recurrence-free patients. In the control set, comprising 65 pre-TUR samples and 29 recurrence-free samples, 72% of all recurrences were detected with the BC MS-MLPA, with a specificity of 55%. Interestingly, all high-grade recurrences were identified. We included urine samples derived from recurrence-free patients as negative controls, since we believe this reflects true clinical practice. Low specificity is observed for all biomarkers that have been investigated for follow up of NMIBC patients and is due to a variety of reasons such as the anticipatory effect. This will be discussed further below under chapter 6.

In **chapter 5**, we aimed to increase recurrence detection by combining multiple molecular assays, i.e. *FGFR3*, *PIK3CA*, *RAS* mutation assays, BC MS-MLPA and microsatellite analysis (MA). All assays previously proved their value as single assay for recurrence detection in voided urine.[16-18] Urine samples were collected from a large retrospective patient cohort, which was part of the European FP7 UROMOL project. All patients initially presented with low-grade NMIBC and urine samples were collected during follow-up visits. A total of 136 patients were included with 716 urine samples and 552 recurrent tumors during a median follow-up of 3 years. Patients were stratified based on the analysis of the primary tumor or tumor at first visit after study inclusion. Stratification depending on the molecular status of the primary tumor is based on the assumption that recurrent tumors will have similar molecular alterations as the previous initial tumor.[19, 20] Hence, stratification should enhance recurrence detection. *FGFR3* analysis without stratification reached a sensitivity of 49% for recurrence detection. Including only patients with a primary *FGFR3* mutant tumor improved recurrence detection to 66%. Highest rate of recurrence detection was reached by the combination of MA and *FGFR3* analysis (82%). All investigated molecular assays were superior to cytology for identifying recurrences. The addition of *FGFR3* analysis to urine cytology increased sensitivity for detection of recurrent Ta disease from 54% to 75% and for grade 1 disease from 33% to 66%. We presume that recurrences were missed because of their relatively small size. A previous study by Zuiverloon *et al* revealed that sensitivity for recurrence detection by *FGFR3* analysis improved with tumor size, suggesting that the amount of seeded tumor cells increases with tumor volume.[21] They also argue that other urine based-assays depending on the number of tumor cells in urine will never reach a sensitivity of 100% for smaller tumors. They propose that multiple urine sampling would increase recurrence detection. Furthermore, we found relatively low specificity for all single and combined markers. We discussed that the low specificity could be partly due to the anticipatory effect of urine analysis, meaning that urine analysis detects the tumor before it is macroscopically visible by cystoscopy. For example, *FGFR3* mutations are not found in sufficient amounts in normal urothelium.[22] Therefore, the specificity of the *FGFR3* assay should be 100%. Yet, it was only 66% in the current study. We showed that 85% of the 'false' positive *FGFR3* urines developed a recurrence within 12 months. A more extensive deliberation about the high false-positive rate is found under chapter 6.

Although the results were promising, we believe that the investigated assays are not suitable for routine clinical practice. The MA consists of 12 individual polymerase chain reactions per sample, which is too laborious in combination with data analysis and needs automation. In addition, a large number of urine samples will not contain sufficient DNA for the MA. The custom MS-MLPA assay proved not to be very easy to handle in the laboratory and many samples failed, also the amount of DNA required was often prohibitive. For this reasons we decided not to proceed with this technology. Therefore, we decided to investigate new promising urinary assays in combination with the solid *FGFR3* assay in **chapter 6**. In this study we investigated the *FGFR3* analysis in combination with the recently developed *TERT* mutation assay and a methylation assay, which both showed promising results in previous reports.[23, 24] The study was part of the European FP7 UROMOL project and included a multicenter prospective patient cohort comprising 1111 patients with primary or previously diagnosed bladder cancer: 720 patients with primary low-grade NMIBC (LG-NMIBC), 259 patients with primary high-grade NMIBC (HG-NMIBC) and 215 patients with MIBC. A total of 2703 urine samples were collected during the first or regular follow-up visits. The main objective of this study was similar to chapter 5 and involved the validation of urinary assays for the follow-up of LG-NMIBC patients. Urine samples were analyzed for *FGFR3* and *TERT* mutations and the methylation of *OTX*. This three-assay combination reached a sensitivity of 57% for the detection of concomitant recurrences from patients with a primary LG-NMIBC, with a specificity of 59%. Marker performance was superior to urine cytology. The detection rate for the three-assay combination increased with advanced stage and grade, identifying 87% of the T1, 77% of the muscle invasive tumors and 83% of the grade 3 tumors. Surprisingly, we found significant higher sensitivity in samples derived from male patients compared to urine samples from females, suggesting the disturbing influence of benign cells in female urine. As in chapter 5, we tried to improve sensitivity by stratification of the patients based on the molecular status of their tumor. This resulted in a slight improvement of marker performance to a sensitivity of 62% with the three-assay combination. Recurrence detection rate with the three-assay combination was significant higher in patients with primary HG-NMIBC compared to patients with primary LG-NMIBC. (57% vs 62%, p-value<0.05). Likewise, the detection rate of primary NMIBC was higher than the detection rate of recurrent tumors, detecting 81% of the primary LG-NMIBC and 94% of the primary HG-NMIBC. This again emphasizes the difficulties in the development of a sensitive urine test for the detection of low-grade recurrences. The additional value of analyzing multiple urine samples to reduce the chance of missing tumor cells in urine from patients with small low-grade recurrence should be investigated in future studies. Unfortunately, specificity of the three-assay combination was still relatively low. The high false-positive rate of urine analysis for concomitant tumor detection could be explained by several reasons. First, sensitivity of white-light cystoscopy is not optimal, ranging from 46%-80%.[25] Therefore, several urine samples must have been mistakenly considered false-positive. In fact there was a tumor present, but it was missed by cystoscopy. Secondly, as mentioned in chapter 4 and 5, low specificity could be due to the anticipatory effect of urine analysis. We revealed that 87% of the *FGFR3* positive urine samples were followed by

a recurrence within 5 years. Likewise, we found that 79% of the urine samples positive for the combination of assays developed a recurrence within 5 years. Remarkably, most high-stage recurrence were found within a year after urine analysis, proposing the additional value of urine analysis for the detection of high-risk recurrences. Furthermore, the low specificity might be due to our definition of recurrence. We defined recurrence as a histologically proven tumor. Yet, small recurrences are frequently coagulated without the collection of tissue for pathological review. These tumors were not included in our data analysis, which could lead to false-positive urine results. Finally, we would like to mention that a very small proportion of the recurrent tumors will appear in the upper urinary tract. Of course, these tumors are missed by cystoscopy.

This study supports the value of urine analysis as an alternative for cystoscopies in patients with LG-NMIBC and as a valuable addition to the surveillance of patients presenting with HG-NMIBC. A study by van der Aa *et al* showed that the knowledge of a positive urine test could improve the awareness of the urologist and thereby increase sensitivity of cystoscopy.[26] Moreover, the next follow-up visit could be adjusted to a shorter time-interval in case cystoscopy is negative. It would be very interesting to construct a recurrence-risk predictor based on urinary assays in combination with clinical and pathological findings.

Risk assessment in patients presenting with hematuria

Patients presenting with microscopic or macroscopic hematuria form a large part of the urological practice.[27] Most important in evaluation of these patients is to discriminate between a malignant and non-malignant cause. Therefore, these patients will undergo invasive examination, including cystoscopy, cytology and imaging of the upper urinary tract. In **chapter 7** we aimed to investigate the predictive value of a urine-based methylation assay in combination with clinical characteristics for the detection of bladder cancer in patients presenting with hematuria. We included 169 patients presenting with microscopic or macroscopic hematuria in which n=54 the cause was malignant. Urine samples were collected at first visit or prior to resection of the primary tumor. Methylation analysis was done for *OSR1*, *SIM2*, *OTX1*, *MEIS1* and *ONECUT2*. The methylation results were combined with clinical variables (i.e. age, gender and type of hematuria) in a multivariable logistic regression model in order to calculate the combined predictive effect. This model determined type of hematuria, *ONECUT2*, *OSR1* and *SIM2* as independent predictors for the presence of BC. We presumed that the addition of smoking status could have further increased diagnostic accuracy, since previous studies showed the independent predictive value of smoking in the assessment of patients presenting with hematuria.[28-30] Unfortunately, smoking data were not available in this study. Next, we determined a cut-off to discriminate between low-risk and high-risk patients. This resulted in 44/54 (82%) of the patients with BC in the high-risk group and 94/115 (82%) of the patients without a malignancy in the low-risk group. All patients with BC assigned to the low-risk group presented with a Ta-tumor and were mostly low-grade. In the current clinical practice, all patients with hematuria are evaluated according the same protocol, without considering the a priori chance according to clinical features. We concluded that our model might be a useful tool in the stratification of patients presenting

with hematuria, resulting in adjustment of patient examination according to patient's risk, and subsequently reduction of costs and patient burden. Several other studies revealed the additional value of molecular markers and clinical characteristics in the evaluation of hematuria.[31-33] Recently, Lotan *et al* validated a nomogram based on clinical characteristics in combination with the FDA-approved NMP22 bladderchek test for risk prediction in a large prospective multicenter patient cohort presenting with hematuria.[31] They again emphasized the importance of including molecular tests in combination of clinical characteristics, such as gender, age and smoking, in decision models in order to avoid extensive evaluation in low-risk patients.

Molecular make-up of bladder cancer under the age of 20: a rare and biological distinct group?

In **chapter 8** and **9** we investigated the molecular status of tumors developed under the age of 20th. Normally, bladder cancer is a disease of the elderly, and is very rare in children with estimated incidence rates of 0.1-0.4%.[34] These bladder tumors are mostly low stage and grade, and they are unlikely to recur. Conflicting results have been published regarding the molecular behavior of bladder cancer in younger patients. Yet, multiple studies showed that tumors of patients <20yr lack the common molecular aberrations as observed in the elderly, like *FGFR3* mutations and hypermethylation of genes associated with bladder cancer in the elderly.[35] These tumors seem to act through a different pathway and form a biologically distinct group compared to their older counterparts. In **chapter 8** we focused on hypermethylation of polycomb group (PcG) target genes and their potential role in bladder carcinogenesis. These genes play an important role in cell lineage decisions in early embryogenesis and are frequently hypermethylated in multiple carcinomas.[36] We hypothesized that hypermethylation of these PcG target genes might be an early event in bladder carcinogenesis and therefore could also be observed in bladder cancer of young patients. Five PcG target genes were selected from a previous genome-wide methylation study[6], i.e. *MEIS1, ONECUT2, OTX1, PCDH7* and *SOX21,* and a total of 167 patients with BC were included. These patients were divided in four different age groups, including <20 years n=14, 20-40yr n=48, 40-60 n=47 and >60yr n=58. Investigation of the tumor DNA revealed significant more methylation in the older age groups compared to the younger ones for 4/5 genes. Only, *PCDH7* showed similar methylation ratios in all four age groups and low methylation in the negative controls i.e. urine from healthy controls. Therefore, we concluded that methylation of the *PCHD7* gene may contribute to early bladder carcinogenesis. Only two studies previously reported about the role of *PCDH7*/ PCDH7 in bladder cancer. Djyrskøt *et al* found PCDH7 underexpression in 60 bladder tumors.[37] Along the same line, Sanchez-Cabayo et al found downregulation of PCDH7 in 105 bladder tumors compared to tissue from 52 healthy controls. In our study, no functional experiments were performed.[38] Yet, it is known that hypermethylation often results in gene silencing. To further investigate the role of PCDH7 in bladder carcinogenesis, functional studies are indicated. In **chapter 9** patients with BC <20yr were screened for oncogenic mutations frequently observed in bladder tumors from the elderly i.e. *FGFR3*, *PIK3CA* and *RAS* mutations. Previously reported data about the mutation

status of bladder cancer in this young age group is very limited. We included 14 patients <20yr and in order to compare the results we included three different control group, similar to those in chapter 8: 20-40 years (n=43), 40-60 years (n=45), and >60 years (n=58). Tumor DNA was analysed for *FGFR3*, *PIK3CA* and *RAS* mutations. None of the 14 included tumors harbored a mutation for *PIK3CA* and only one tumor showed a *FGFR3* mutation. Interestingly, in 4/14 (29%) patients <20yr a *HRAS* mutation was found. In contrast, only 13% (19/146) of the patients in the older control groups harbored a *RAS* mutation. Even more remarkable was the fact that 3/4 observed *HRAS* mutations found in the youngsters were rarely described in tumors of the elderly i.e. c.34G>A (p.(Gly12Ser)) and c.35C>G (p.(Gly12Ala). Another c.35C>G *HRAS* mutation was found in a bladder tumor of a 29yr-old patient from the control group. This patient was known to have the Costello Syndrome, a rare congenital disorder caused by germline mutations in the *HRAS* gene, with c.35C>A/G as most observed mutation. Based on these findings we hypothesized that the other four patients could be mosaic for the *HRAS* mutation and therefore display some of the clinical features of the Costello Syndrome such as tumor predisposition. In order to strengthen this hypothesis, normal surrounding bladder tissue was analysed for the *RAS* mutations which revealed similar *HRAS* mutations in the 29yr-old Costello patient, but also in 2/4 of the other patients. Based on these results we concluded that these two patients could be mosaic for the *HRAS* mutation. The analysis of blood is necessary to fully prove this hypothesis. Unfortunately, this was not possible within the current study set-up due to ethical limitations. Hafner *et al* previously described *HRAS* mosaicism in relation to bladder cancer in a case report.[39] In this report, a 49yr-old patient is described with a congenial epidermal naevus and bladder cancer at the age of 19 which recurred after a 29yr recurrence-free interval. One year later a single lung metastasis was found. *RAS* mutation analysis revealed a p.Gly12Ser *HRAS* mutation in the epidermal naevus in the bladder tumors and lung metastasis, but also in the normal lung tissue, blood leucocytes and normal urothelium. No other features of the Costello Syndrome were observed. The authors concluded that these results support the theory that *RAS* mutations might occur during early embryogenesis, leading to mosaicism. Eventually, this could contribute to the development of cancer during life. Our results support these findings and suggest that the chance of developing bladder cancer at an early age is increased. Therefore, we believe that tumors from patients presenting with bladder cancer at a young age should be screened for *HRAS* mutations. In case of a mutation further investigation is recommended.

Future perspectives and concluding remarks

Non-muscle invasive bladder cancer is a unique type of cancer with an overall favorable prognosis, despite the high recurrence rate and chance of disease progression. Many studies investigated the identification of new molecular markers to facilitate the intensive surveillance protocol and thereby improve costs and patient burden. In order to reach this goal it is crucial to precisely select patients suitable for urine surveillance. This group includes patients with a low risk of disease progression. Currently, this progression risk is still highly dependable on the pathological characteristics, even though there is a huge interobserver and intraobserver variability. We

composed a new molecular grade to objectify tumor aggressiveness. We believe that this new molecular grade could improve progression prediction and therefore help us to stratify patients suitable for less invasive follow-up. This new molecular grade is currently being validated in a large prospective patient series. When this is successful, the test could be incorporated in the routine diagnostic work-up of patients presenting with pTa bladder cancer.

Up to now, no urinary assays are included in the guidelines, since the marker performance is deemed not to be high enough to safely reduce the amount of cystoscopies. One of the reasons, besides the sensitivity of the markers, is that one assumes that cystoscopy, the gold standard, has a sensitivity of 100%. Realizing that this is overoptimistic is important to rethink the contribution that biomarkers can have in the follow-up of patients. In this thesis we combined several urinary tests in order to improve recurrence detection and proved that a combination of assays is sensitive for the detection of the most aggressive tumors. Yet, the detection of small low-grade recurrence remains challenging. Therefore we emphasize the need of multiple urine sampling to improve recurrence detection rate. Recently, a large multicenter randomized control trial (RCT) has started at the Erasmus Medical Center, Rotterdam. Patients with primary or recurrent NMIBC are included and double urine samples will be collected at each follow-up visit. Samples are investigated with the combination of markers discussed in this thesis. The main objective of the RCT is to prove that it is possibly to safely reduce the number of cystoscopies during follow-up in patients with a low-risk NMIBC. Furthermore, the study will also investigate the additional value of urine analysis in the follow-up of patients with high-risk NMIBC, assuming that urine analysis is able to earlier detect potential dangerous recurrences. Expectantly, results of this RCT will be able to change current bladder cancer follow-up. As future perspective, we advocate a urine-first strategy, meaning that the analysis of urine samples will reduce the number of cystoscopies, since only a positive urine test will be followed by cystoscopy. This will be mostly relevant for patients with low-risk pTa-tumors, especially because the sensitivity of cystoscopy is relatively low for the detection of these pTa-tumors. Another possible development is the use of next generation sequencing on urinary DNA. With this technology multiple genes can be sequenced simultaneously using only a small amount of DNA. For instance it would be possible to include tumor suppressor genes such as *TP53* and *KDM6A,* both frequently mutated in BCs.[40]

Furthermore, we presume that urinary markers could not only play a role in bladder cancer surveillance, but also in the initial diagnosis. Hematuria patients form a large part of the urological population and unnecessary invasive investigation can be avoided by proper risk stratification based on an a priori chance calculated using clinical variables in combination with urine analysis. We proposed such a risk model in this thesis, which is currently validated in a larger group of patients.

As final remark, I would like to stress out that molecular analysis is justified in bladder tumors from patients <20yrs, even though the overall prognosis of this age group is favorable and

genetic aberrations are uncommon. We showed an unexpected high percentage of *RAS* mutations in this age group and assume that this could be a sign of mosaicism. These patients need further clinical evaluation.

In summary, bladder cancer is a fascinating disease and there is a big role for molecular markers in the development, diagnosis and prognosis of this disease. In this thesis we investigated molecular markers in relation to these three different facets with improvement of patient care as ultimate goal. Stratification of patients prior to initial diagnosis as well as during follow-up in combination with surveillance by urinary markers will lead to less invasive investigations, resulting in reduction of costs and patient burden.

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Chapter 11

Summary/Samenvatting

SUMMARY

Bladder cancer is the 5th most common cancer in the western world. It could simply be divided into two different subgroups: non-muscle invasive BC and muscle-invasive BC. 75% of all BC patients will have non-muscle invasive disease at initial presentation. This type of BC has a favorable prognosis, although it is characterized by a high recurrence rate and a chance of progression into muscle invasive disease with much worse prognosis. Hence, these patients should be followed extensively by cystoscopy. Cystoscopy is an invasive unpleasant procedure causing patient discomfort. Furthermore, the frequent surveillance in combination with the high number of TUR's results in high costs, making BC one of the most expensive cancer types. Using non-invasive tests for the detection and follow-up of BC patients could reduce costs and reduce patients burden. Moreover, molecular tests could be used for risk stratification by predicting prognosis. This could contribute to adjustment of the currently used surveillance protocols. In this thesis we studied the use of molecular markers in relation to these aspects. Furthermore, we investigated a particular subgroup of BC patients, namely patients with BC under the age of 20 years. BC in this age group is very rare and therefore there is less known about the molecular characteristics in this specific patient group.

At first a short introduction is given about bladder cancer in **chapter 1**. It includes epidemiology, symptoms and diagnosis and the pathological assessment. Also treatment options and the use of molecular markers are discussed, followed by the scope of the thesis.

The second part includes chapter 2 and chapter 3 and investigates molecular markers in relation to tumor progression. **Chapter 2** is a validation of the previously identified Ta-progression markers *GATA2*, *TBX2*, *TBX3*, *ZIC4* in a large set of patients with primary Ta-BC. 77/192 of the included patients developed disease progression to muscle-invasive disease. The methylation results were combined in a Cox-regression analysis and showed the independent effect of *TBX2*, *TBX3*, *ZIC4* on progression risk. Based on the best predictors *TBX2* and *TBX3* new molecular grades were defined. Progression-free survival was significant better in patients with low molecular grade compared to the higher molecular grades. Likewise, patients with intermediate molecular grade showed better progression-free survival compared to the high molecular grade group. This new molecular grade could be valuable for objective assessment of tumor aggressiveness. **Chapter 3** investigated the prevalence of *TERT* mutations in BC and found that 79% of all BC harbored a *TERT* mutation independent of tumor stage. Most frequently observed mutations were C228T and C250T. There was no association with age, sex of smoking status. Furthermore, there was no relation between *TERT* mutation status and progression-free survival, disease-specific survival or overall survival. Urine analysis with the *TERT* SNaPshot assay revealed sensitivity for recurrence detection of 42% and 62% for the detection of the initial tumor. Specificity was 90% in patients without a history of BC and 73% for patients in follow-up for BC.

In the third part we investigate the role of urine-based tests for primary diagnosis and follow-up of patients with NMIBC. Previous studies showed the diagnostic ability of methylation analysis for detection of BC in voided urine. In **chapter 4** we designed a methylation analysis specifically for the detection of recurrent NMIBC. Genes methylated in recurrent NMIBC were selected for the BC-specific methylation analysis (BC MS-MLPA) comprising 23 different probes from 12 different genes. Next, the assay was validated on urine from an independent set of patients with recurrent disease and urine from healthy controls. Based on these results a four-gene panel was selected including *APC_a*, *TERT_a*, *TERT_b*, and *EDNRB*. This panel reached an AUC of 0.82 in a test-set of patients and 0.69 in a validation set of patients. The sensitivity of this four-gene panel for the detection of recurrent BC in urine was 63% in the test-set and 72% in the validation-set, with specificity of 58% and 55%, respectively. Interestingly, most high-grade tumors (10/11) were detected with this BC-specific methylation assay. In **chapter 5**, this methylation assay was combined with other molecular assays (*FGFR3*, *PIK3CA* and *RAS* mutation analysis, MA) in order to improve sensitivity for recurrence detection. Patients with primary low-grade NMIBC were included and were stratified based on the molecular status of the primary tumor. *FGFR3* analysis detected 66% of all recurrent tumors. The highest recurrence detection rate was observed with a combination of *FGFR3* analysis and MA (82%). All molecular assays and combination of assays were superior to cytology. The addition of *FGFR3* analysis to urine cytology improved sensitivity from 56 % to 76%. Overall the specificity was low, ranging from 34%-66%. Since the BC MS-MSLPA and MA showed some flaws, we combined the *FGFR3* analysis with two other promising molecular assays in **chapter 6**. This study was a large prospective multicenter patient cohort including 977 patients with NMIBC. 2496 urine samples were collected at primary visit or during follow-up and were examined with the *FGFR3* and *TERT* mutation analysis in combination with *OTX1* methylation analysis. This three-assay combination reached a sensitivity of 57% for the detection of a concomitant recurrence in patients with primary low-grade NMBIC (LG-NMIBC). The specificity was 58%. The sensitivity increased with advanced stage and grade (87% T1, 77% ≥T2, 83% G3). Sensitivity for recurrence detection in patients with primary high-grade disease was 72%. Furthermore, we showed that a positive urine assay was associated with a recurrence over time in 76% of the cases. Especially high stage recurrences were diagnosed concomitantly or within 1 year after a positive urine assay. This emphasizes the anticipatory effect of urine analysis. This study supports the value of urine analysis as an alternative for cystoscopies in patients with LG-NMIBC and as a valuable addition to the surveillance of patients presenting with HG tumors. In **chapter 7** we investigated whether the MS-SNaPshot, which was developed for recurrence detection, would be able to discriminate between malignant and non-malignant disease in patients presenting with hematuria. Methylation analysis on voided urine was performed for *OSR1*, *SIM2*, *OTX1*, *MEIS1* and *ONECUT2*. Methylation results were combined with clinical variables (i.e. age, gender and type of hematuria) into a logistic regression model to calculate the combined predictive effect. Patients were divided into the low-risk group or high-risk group based on this model. 82% of the patients with BC as cause of hematuria were included in the high-risk group and 82% of the patients with hematuria due to a non-malignant

cause were assigned to the low-risk group. We concluded that risk assessment based on these molecular markers in combination with clinical variables could be useful in the stratification of patients presenting with hematuria.

The final part studies the molecular status of bladder cancer in patients under 20 years old. There is less known about BC in this young age group, but these tumors appearing to form a molecular distinct group. First, we analyzed the tumors from patients <20yrs for methylation of five polycomb group target genes (*MEIS1, ONECUT2, OTX1, PCDH7* and *SOX21*) and compared the results to those from patients with BC in different age groups (**chapter 8**). Four out of five genes showed significant less methylation in the younger patients compared to the older ones. Only *PCDH7* revealed similar high methylation ratios in all four age groups and low methylation ratios in the negative controls. Therefore, we presumed that *PCHD7* might be a possible candidate in early bladder carcinogenesis. **Chapter 9** investigated the mutation status of *FGFR3*, *PIK3CA* and *RAS* in BC from patients <20yr. No *PIK3CA* mutations and only one *FGFR3* mutation were observed. Interestingly a high amount of *HRAS* mutations were found (29%) and 3/4 of the *HRAS* mutations were rarely described in tumors from older BC patients (c.34G>A and c.35C>G). We found another c.35C>G mutation in BC from one of the older patients. This patient was known to have the Costello syndrome. This is a rare congenital disorder and is associated with cancer predisposition like BC. It is caused by *HRAS* germline mutations (mainly c.34G>A and c.35C>G). We analyzed normal surrounding tissue for *HRAS* mutations and found similar *HRAS* mutations in tissue of the Costello patient, but also in 2/4 of the other patients. Based on these results we concluded that these two patients could be mosaic for the *HRAS* mutation and therefore might have some of the clinical features such as tumor predisposition.

SAMENVATTING (DUTCH)

Blaaskanker (BK) is de vijfde meest voorkomende kanker in de westerse wereld. BK wordt over het algemeen in twee verschillende groepen verdeeld: niet-spierinvasief blaascarcinoom (NSIBC) en spierinvasief blaascarcinoom (SIBC). 75% van alle patiënten die zich voor het eerst presenteert met BK heeft NSIBC. Deze vorm van BK heeft een goede prognose, alhoewel dit type wordt gekarakteriseerd door een hoog aantal recidieven en de kans op progressie naar spierinvasieve ziekte met een veel slechtere prognose. Door deze typische karakteristieken moeten patiënten met NSIBC gedurende vele jaren regelmatig worden gecontroleerd door middel van cystoscopie. Dit is een invasieve procedure, die door patiënten vaak als onplezierig wordt ervaren. Deze intensieve manier van surveillance resulteert samen met het vele aantal TUR's in hoge kosten. Dit maakt BK een van de duurste vormen van kanker om te behandelen. Kosten kunnen worden gereduceerd door het gebruik van niet-invasieve tests. Hierdoor kan ook de last voor de patiënt worden verminderd. Verder kunnen moleculaire markers bijdragen aan het stratificeren van patiënten door middel van een risico-inschatting (bijvoorbeeld de kans op progressie). Uiteindelijk zou dit kunnen leiden tot aanpassing en verbetering van het huidige surveillanceprotocol. In dit proefschrift hebben we het gebruik van moleculaire markers in relatie tot deze verschillende aspecten bestudeerd. Daarnaast hebben we een aparte groep van BK-patienten onderzocht. Deze groep bestaat uit patiënten met BK onder de 20 jaar. BK is in deze leeftijdsgroep zeer zeldzaam en daarom is er erg weinig bekend over de moleculaire status van deze tumoren.

Eerst wordt een korte introductie gegeven over blaaskanker in **hoofdstuk 1**. Hierin worden de epidemiologie, symptomen en diagnose, en pathologische beoordeling besproken. Verder wordt er aandacht besteed aan de behandelingsopties en het gebruik van moleculaire markers. Daarna volgt in het kort de strekking van dit proefschrift. Het tweede deel beslaat hoofdstuk 2 en hoofdstuk 3 en onderzoekt de rol van moleculaire markers in relatie tot BK-progressie. **Hoofdstuk 2** bestaat uit de validatie van de eerder geïdentificeerde progressie markers *GATA2*, *TBX2*, *TBX3* en *ZIC4* in een grote set van patiënten met een primair pTa-BK. Veertig procent (77/192) van de geïncludeerde patiënten ontwikkelde progressie naar spierinvasieve ziekte. De primaire tumoren van de patiënten werden geanalyseerd met de vier methyleringsmarkers en de resultaten werden gecombineerd in een Cox-regressie analyse. Deze analyse liet zien dat zowel *TBX2*, *TBX3*, als ook *ZIC4* een onafhankelijk effect hadden op de progressiekans. *TBX2* en *TBX3* waren de beste voorspellers en op basis van deze twee methyleringsmarkers definieerden we een nieuwe moleculaire gradering. De progressie-vrije overleving was significant beter in patienten met een laag moleculair gegradeerde tumor ten opzichte van tumoren met een intermediaire of een hoge moleculaire gradering. Het zelfde gold voor tumoren met een intermediaire gradering in vergelijking tot tumoren met een hoge moleculaire gradering. Wij denken dat deze nieuwe moleculaire gradering goed bruikbaar is om de mate van tumoragressiviteit te bepalen. Mogelijk kan in de toekomst het surveillanceprotocol hierop aangepast worden.In **hoofdstuk 3** werd de prevalentie van *TERT* mutaties in BK onderzocht en vonden we dat 79% van alle BK,

onafhankelijk van het tumor stadium, een *TERT* mutatie had. De twee meest voorkomende *TERT* mutaties waren C228T en C250T. Er was geen associatie met leeftijd, geslacht, of roken. Verder was er geen relatie tussen *TERT* mutatie status en de progressie vrije overleving, ziekte-specifieke overleving, of totale overleving. De sensitiviteit voor het detecteren van een recidief in urine door middel van de TERT SNaPshot analyse was 42% en voor het detecteren van een primaire tumor was deze 62%. De specificiteit was 90% voor personen zonder een historie van BK en 73% voor patiënten met een verleden van BK.

In het derde deel wordt ingegaan op het gebruik van urinediagnostiek voor het stellen van de initiële diagnose of voor de follow-up van patiënten met NSIBC. Eerdere studies hebben aangetoond dat analyse van urine met methyleringsmarkers gebruikt kan worden voor het detecteren van BK. In **hoofdstuk 4** hebben we een methylatie-assay (BC MS-MLPA) ontwikkeld speciaal voor het detecteren van recidiverend NSIBC. Voor dit methylatie-assay werden er genen geselecteerd die gemethyleerd zijn in recidiverend NSIBC. Dit assay bestond uiteindelijk uit 23 verschillende probes voor 12 verschillende genen. Dit assay is vervolgens gevalideerd op urine van een onafhankelijke groep patiënten met recidiverend BK. Urines van gezonde patiënten fungeerden als controle. Op basis van deze resultaten werden er vier genen geselecteerd (*APC_a*, *TERT_a*, *TERT_b*, en *EDNRB*). Dit 4-genenpanel behaalde een AUC van 0.82 in de testgroep en 0.69 in de validatiegroep. In de testgroep was de sensitiviteit voor het detecteren van recidiverend BK 63% en in de validatiegroep was dit 72%. De specificiteit was respectievelijk 58% en 55%. Verrassend genoeg werden bijna alle hooggradige tumoren (10/11) gedetecteerd met dit 4-genenpanel. In **hoofdstuk 5** werd de BC MS-MLPA gecombineerd met andere moleculaire tests (*FGFR3*, *PIK3CA*, *RAS* mutatie analyse, MA) om zo de sensitiviteit voor het detecteren van recidieven te vergroten. Patiënten werden geïncludeerd met een primair laaggradig NSIBC en gestratificeerd op basis van de moleculaire status van hun primaire tumor. *FGFR3* analyse detecteerde 66% van alle tumorrecidieven. Het hoogste aantal recidieven werd gedetecteerd door middel van de combinatie MA en *FGFR3* analyse (82%). Alle individuele markers en combinaties van assays waren beter in het detecteren van recidieven ten opzichte van cytologie. De toevoeging van *FGFR3* analyse aan cytologie resulteerde in een verbetering van de sensitiviteit van 56% naar 76%. De specificiteit was in alle gevallen vrij laag, variërend van 34% tot 66%. Omdat zowel de BC MS-MLPA als de MA enkele gebreken vertoonde, hebben we besloten om in **hoofdstuk 6** de *FGFR3* analyse met twee andere veelbelovende urine-assays te combineren. Deze studie betrof een groot prospectief multicenter cohort met 977 patiënten geïncludeerd met NSIBC. 2496 urines werden verzameld bij eerste polibezoek of gedurende follow-up. De urine werd vervolgens geanalyseerd met de *FGFR3* en *TERT* mutatuie-analyse en de *OTX1* methylatie-analyse. Deze combinatie van drie assays resulteerde in een sensitiviteit van 57% voor de detectie van een gelijktijdig recidief in patiënten met een primair laaggradig NSIBC. De specificiteit was 58%. De sensitiviteit verbeterde naar mate het stadium of de gradering van het recidief toenam (87% T1, 77% ≥T2, 83% G3). Dezelfde combinatie van assays detecteerde 72% van de recidieven in patiënten met een primair hooggradig NSIBC. Daarnaast toonden

we aan dat 76% van de positieve urines binnen 5 jaar werd gevolgd door een recidief en dat recidieven met een hoog stadium bijna allemaal binnen 1 jaar na positieve urine-analyse werden gediagnosticeerd. Dit benadrukt het voorspellende effect van urinediagnostiek. In deze studie lieten we zien dat met een combinatie van urine-assays de detectiekans van een recidief wordt vergroot. Voor patiënten met een primair laaggradig NSIBC kan urinediagnostiek dienen als goed alternatief voor cystoscopie. Daarnaast kan urinediagnostiek in verband met zijn voorspellende waarde van aanvullende betekenis zijn in de follow-up van patiënten met primair hooggradig NSIBC. In **hoofdstuk 7** van dit proefschrift onderzochten we of de MS-SNaPshot, die in eerste instantie ontwikkeld was voor het detecteren van BK recidieven, ook ingezet kan worden voor het diagnosticeren van BK in patiënten die zich presenteerden met hematurie. Urine werd geanalyseerd met de MS-SNaPshot voor de genen *OSR1*, *SIM2*, *OTX1*, *MEIS1* en *ONECUT2*. Deze resultaten werden samen met klinische variabelen (leeftijd, geslacht en type hematurie) gecombineerd in een logistisch regressie model om zo de voorspellende waarde voor aanwezigheid van een maligniteit te berekenen. Op basis van het model werden de hematuriepatienten verdeeld tussen een hoogrisico- en een laagrisicogroep. 82% van de patiënten met BK als oorzaak van de hematurie zaten in de hoogrisicogroep en 82% van de patiënten met een niet-maligne oorzaak van de hematurie werden aan de laagrisicogroep toebedeeld. Wij concludeerden dat risico inschatting op basis van moleculaire markers in combinatie met klinische variabelen van belang kunnen zijn voor het stratificeren van patiënten die zich presenteren met hematurie. Hierdoor kunnen onnodige onderzoeken in laagrisicopatienten worden voorkomen.

Het laatste deel van het proefschrift bestudeert de moleculaire status van BK in patiënten jonger dan 20 jaar. Er is weinig bekend over BK in deze jonge leeftijdsgroep, maar het lijkt erop dat tumoren in deze groep zich moleculair apart gedragen ten opzichte van BK in oudere leeftijdsgenoten. Eerst hebben we tumoren van deze jonge patiënten geanalyseerd op de aanwezigheid van methylering van vijf zogenaamde polycombgroep targetgenen (*MEIS1, ONECUT2, OTX1, PCDH7* en *SOX21*). Vervolgens hebben we de resultaten vergeleken met de resultaten van oudere patiënten met BK (**hoofdstuk 8**). Vier van de vijf genen toonden significant minder methylatie in de tumoren van de jongere patiënten vergeleken met de ouderen. Alleen *PCDH7* toonde vergelijkbare methyleringsratio's in alle leeftijdsgroepen. Tevens werden er lage methyleringsratio's gevonden in urine van de gezonde controle patiënten. Daarom concludeerden we dat *PCDH7* mogelijk een rol zou kunnen spelen in de vroege blaascarcinogenese. **Hoofdstuk 9** onderzoekt de mutatiestatus van *FGFR3*, *PIK3CA* en *RAS* in dezelfde leeftijdsgroep. Er werden geen *PIK3CA* mutaties gevonden en 1 *FGFR3* mutatie. Echter werden echter wel een hoog aantal *HRAS* mutaties gevonden (29%) en 3/4 HRAS mutaties waren zelden beschreven in BK van oudere patiënten (c.34G>A en c.35C>G). Er werd ook eenzelfde c.35C>G mutatie gevonden in 1 van de patiënten in de controlegroep. Deze patiënt bleek het Costellosyndroom te hebben. Dit is een zeer zeldzaam aangeboren syndroom, dat onder andere gekenmerkt wordt door een predispositie voor het ontwikkelen van tumoren (o.a. blaaskanker). Dit syndroom wordt veroorzaakt door kiemlijn mutaties in *HRAS* (voornamelijk c.34G>A en c.35C>G). Hierop

besloten wij om ook normaal omliggend weefsel te analyseren op de aanwezigheid van deze *HRAS* mutaties. Zoals verwacht werd er ook een *HRAS* mutatie in het gezonde weefsel van de Costello patiënt gevonden. Daarnaast toonden 2 van de 4 van de jonge patiënten met een *HRAS* mutatie in de tumor ook een *HRAS* mutatie in het gezonde weefsel. Gebaseerd op deze resultaten concludeerden we dat deze patiënten mogelijk mozaïek zouden kunnen zijn voor deze *HRAS* mutatie en daardoor mogelijk enkele verschijnselen van het Costellosyndroom kunnen vertonen, zoals tumorpredispositie.

PART VI

APPENDICES

LIST OF ABBREVIATIONS

AUC	Area Under the Curve
BC	Bladder Cancer
BCG	Bacillus Calmette-Guérin
CIS	Carcinoma In Situ
EAU	European Association of Urology
EDNRB	Endothelin Receptor Type B
EORTC	European Organization for Research and Treatment of Cancer
FGFR	Fibroblast Growth Factor Receptor
LOH	Loss Of Heterozygosity
MA	Microsatellite Analysis
MIBC	Muscle-Invasive Bladder Cancer
MS-MLPA	Methylation-Specific Multiplex Ligation-dependent Probe Amplification
NMIBC	Non-Muscle Invasive Bladder Cancer
ONECUT	One Cut Homeobox
OSR	Odd-Skipped Related
OTX	Orthodenticle homeobox
PCDH	Protocadherin
PCG	Polycomb group target genes
PIK3CA	Phosphatidylinositol-3,4 -bisphosphate 3-kinase
TERT	Telomerase Reverse Transcriptase
TUR	TransUrethral Resection
WHO	World Health Organization

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Willemien Beukers was born on the 24th of January 1986 in Rotterdam, the Netherlands. After finishing secondary school at the Johan de Witt Gymnasium in Dordrecht in 2004, she started studying Medicine at the Erasmus University Rotterdam, the Netherlands. Beside her study, she worked as a research student at the department of Pathology and participated in the Zwarthoff-group investigating urinary diagnostics in bladder cancer. In 2009 she obtained her Master degree in medicine and continued her research at the department of pathology as PhD-student under supervision of prof.dr. Ellen C. Zwarthoff. She started her clinical rotations in 2013 and graduated as medical doctor in 2015. After her graduation, she worked a couple of months as a surgical resident at the Spaarne Gasthuis in Haarlem, the Netherlands. She recently started as urological resident at the Erasmus Medical Center in Rotterdam. She aims to start with the urology training in 2017.

OVER DE AUTEUR

Willemien Beukers werd geboren op 24 januari 1986, te Rotterdam. Na het behalen van het Gymnasiumdiploma aan het Johan de Witt Gymnasium, te Dordrecht, begon zij in 2004 met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Naast haar studie deed zij onderzoek naar urinediagnostiek in blaaskanker op de afdeling pathologie onder begeleiding van prof. dr. E.C Zwarthoff en dr T.C.M. Zuiverloon. In 2009 behaalde zij haar doctoraal geneeskunde en kon hierna haar werk op de afdeling Pathologie voortzetten in het kader van een promotietraject. In maart 2013 hervatte zij haar studie geneeskunde en behaalde in 2015 haar artsdiploma. Na het behalen van haar artsexamen werkte zij voor een aantal maanden als ANIOS chirurgie in het Spaarne Gasthuis, te Haarlem. Recent is zij gestart als ANIOS urologie in het Erasmus MC, te Rotterdam. In 2017 hoopt zij te starten met de opleiding tot uroloog.

PHD PORTFOLIO

Name PhD student	Willemien Beukers
Erasmus MC department	Pathology
PhD period	2009-2015
Promotor	Prof. dr. E.C. Zwarthoff

Courses |	ECTS
2009 Basic and Translational Oncology	1.8
The Erasmus Postgraduate School of Molecular Medicine, Rotterdam, the Netherlands	
2009 Advanced imaging techniques for medical doctors	0.2
The Erasmus Postgraduate School of Molecular Medicine, Rotterdam, the Netherlands	
2009 Molecular diagnostics for Medical Doctors	0.2
The Erasmus Postgraduate School of Molecular Medicine, Rotterdam, the Netherlands	
2010 Basic Course Oncology, Ellecom, the Netherlands	1.6
Nederlandse Vereniging voor Oncologie	
2010 Classical methods in data analysis	5.7
Netherlands Institute for Health Sciences, Rotterdam, the Netherlands	
2012 Biomedical English Writing and Communication	2.0
The Erasmus Postgraduate School of Molecular Medicine, Rotterdam, the Netherlands	

Oral/Poster presentations |	
2010 Dutch Urology Society (NVU), biennial meeting. *Nieuwegein, the Netherlands*	0.3
2010 Meeting for Experimental Surgical Research (SEOHS). *Rotterdam, the Netherlands*	0.3
2011 Postgraduate School of Molecular Medicine day. *Rotterdam, the Netherlands*	0.3
2011 European Association of Urology, annual meeting. *Vienna, Austria*	1.0
2011 Dutch Association of Pathology (NVVP). *Zeist, the Netherlands*	0.3
2012 European Association of Urology, annual meeting. *Paris, France*	1.0
2012 American Urology Association, annual meeting. A*tlanta, USA*	1.0
2012 EAU Section on Urological Research, annual meeting. *Strasbourg, France*	1.0
2013 European Association of Urology, annual meeting. *Milan, Italy*	1.0
2013 American Association Cancer Research, annual Meeting. *Washington D.C, USA*	1.0

International conferences |	
2011 European Association of Urology, annual meeting. *Vienna, Austria*	1.0
2012 European Association of Urology, annual meeting. *Paris, France*	1.0
2012 American Urology Association, annual meeting. *Atlanta, USA*	1.0
2012 EAU Section on Urological Research, annual meeting. *Strasbourg, France*	1.0

Seminars |

2009-2012 Josephine Nefkens Institute presentations	2.5
2009-2012 Research group lectures – Zwarthoff-group	2.5
2009-2012 PhD-meetings department of Urology	1.0

Teaching Activities |

Supervision third year medical students 'keuzeonderwijs', *2009*	1.6
Supervision second year medical students 'keuzeonderwijs', *2009-2011*	0.9
Schooling of OR-nurses: bladder surgery, *2011*	0.3
Junior science program, *2011*	1.6
Junior science program, *2012*	1.6
Supervision of students:	
Diandra van Houwelingen, student applied sciences	4.0
Jie-Fen Chin, medical student	4.0
Hrvoje Kovacic, medical student	4.0
Aleksander Herzegovac, medical student	4.0

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In 2008 begon het voor mij allemaal in het Zwarthoff-lab als onderdeel van een klein studentenimperium dat dr. Zuiverloon had opgebouwd. Ruim een jaar later mocht ik haar plek overnemen en begon ik aan dit proefschrift. Een lange tijd ben ik dus onderdeel geweest van de Zwarthoff-groep. Een tijd waarin ik veel heb geleerd, ben gegroeid, en heel veel fijne mensen om me heen heb gehad. Dus nu is het mijn beurt om jullie allemaal te bedanken.

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