Molecular Diagnosis in Bladder Cancer

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The research described in this thesis was performed at the Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands, and was financially supported by the following grants: Koningin Wilhelmina Fonds 2006-3672 and European Union (FP7, project 201663, UROMOL)

Cover: Artist impression of DNA extracted from bladder tumor cells in voided urine

Cover Design: Carmen van Thol, my mother

Layout/Print: Dopas Productions

ISBN: 978-90-9027678-6

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Molecular Diagnosis in Bladder Cancer

Moleculaire diagnostiek bij blaaskanker

Proefschrift

Ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het college van promoties.

De openbare verdediging zal plaatsvinden op
woensdag 4 september 2013 om 09:30 uur.

door

Tahlita Carmelita Margarita Zuiverloon
Geboren te Haarlem

PROMOTIECOMMISSIE

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It's true
Life will smile for you
It's true
Life will smile for you

You thought you knew it all
But let me tell you
You're not the only one by far
Let's knock down all these walls
Take the first step and you will get there in time

It's true
Life will smile for you

Axwell & Sebastian Ingrosso
vs. Salem Al Fakir 2007

Voor mijn ouders

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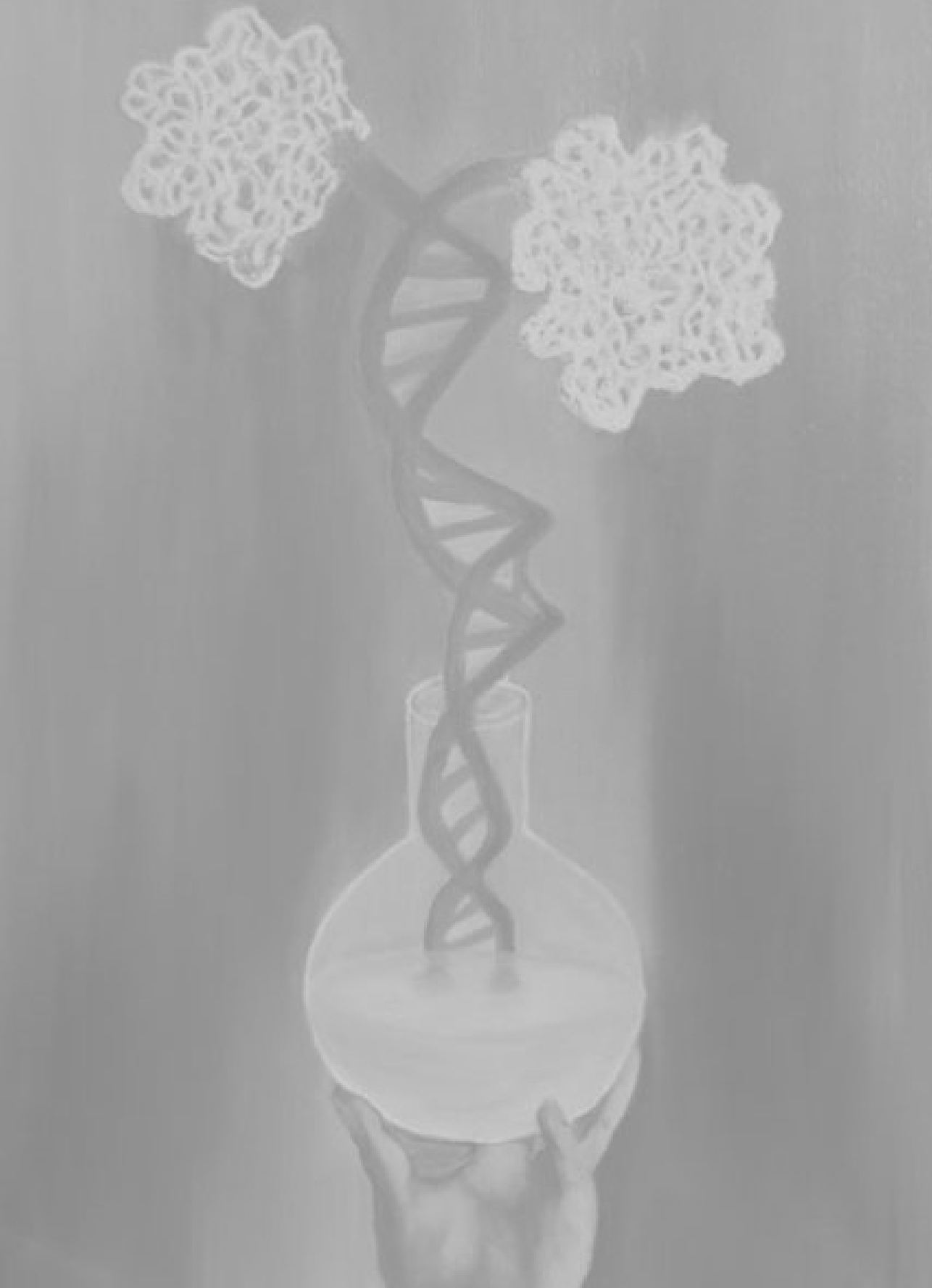


PART I

GENERAL INTRODUCTION

Chapter 1

Introduction and scope of the thesis

1. Introduction

1.1 Epidemiology

Bladder cancer (BC) is the most prevalent type of urothelial cancer and is associated with the highest costs of all cancer types due to intensive patient surveillance. Because bladder tumors frequently recur, patients need to be monitored extensively [1-4]. Incidence increases with age with the highest frequency between the ages of 60-70 years.

The strongest risk factors associated with BC are smoking, occupational exposure to carcinogens and chronic infection of the *Schistosoma haematobium* parasite [5].

Recent genome-wide association studies identified multiple loci to be associated with the susceptibility to BC [6, 7]. Mostly men are affected at a ratio of 3:1 and this is mainly explained by the difference in smoking behavior [8].

Histological subtypes of bladder cancer include urothelial cell carcinoma (90%), squamous cell carcinoma (6-8%) and adenocarcinoma (1-2%). Urothelial cell carcinoma originates from the epithelial layer of the bladder (urothelium). Squamous cell carcinoma is mainly found in developing countries and associated with chronic infection of S. haematobium.

1.2 Symptoms and diagnosis

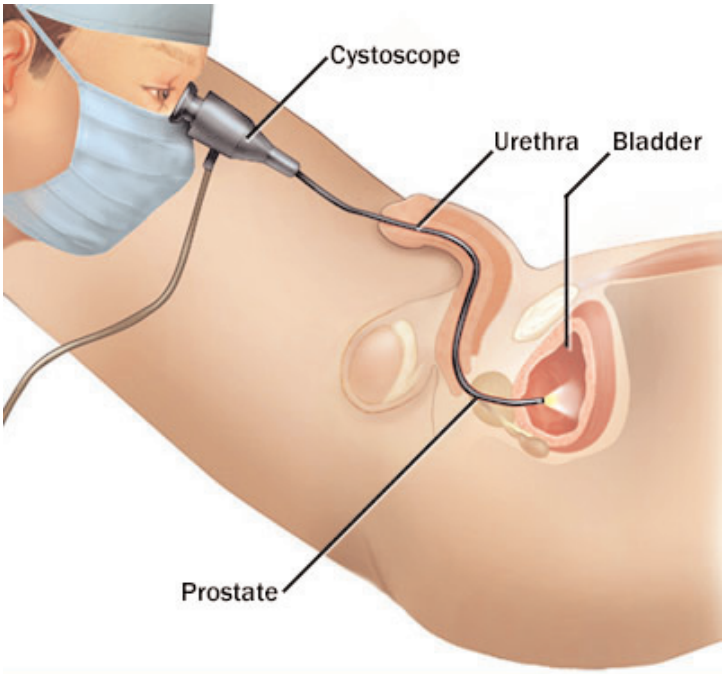
The most important symptom of bladder cancer is painless micro- or macroscopic hematuria. Less frequently observed symptoms include frequency, urgency (sudden urge to urinate) and dysuria (pain during urination), but these symptoms are not specifically associated with bladder tumors and are also found in cystitis, prostatitis or bladder stones.

When patients present at the urologist with these complaints, the presence of hematuria will first be investigated. Additionally, urine cytology is performed. The definitive diagnosis is made through endoscopic investigation of the bladder by using a cystoscope (Figure 1a).

A cystoscope consists of a light source and a tube containing either lenses or optical fibers to transport an image recorded at the tip of the instrument to a monitor. A rigid or flexible scope can be used depending on the purpose of the investigation. The cystoscope is placed into the urethra until the bladder is reached. The bladder is filled with water and investigated. Originally only white-light was used during cystoscopy, but over the years some disadvantages became apparent and lead to the development of new techniques. It is not possible to determine the histologic stage and grade of a tumor with classic white-light cystoscopy and this necessitates a second investigation. Additionally, white-light cystoscopy has a low sensitivity for the detection of carcinoma in situ (CIS). More recently, photodynamic diagnosis (PDD) was developed to improve cystoscopic detection of bladder tumors. During PDD fluorescence (blue light) is used. Prior to investigation, a photosensitizer ((hexi)-5-aminolaevulinic acid, 5-HAL or 5-ALA) is instilled into the bladder for one hour and the cystoscopy is carried out within two hours after emptying the bladder. Multiple studies demonstrated a higher sensitivity of PDD than white-light cystoscopy for the detection of papillary lesions and CIS, but the low specificity still remains a problem (Figure 1b) [9-11]. Narrow band imaging (NBI) is a new technique that does not require administration of an exogenous contrast agent. The technique uses different wave lengths (narrow bandwidths) strongly absorbed by hemoglobin, thus enhancing the contrast between bladder mucosa and vascular structures. In the same line, more vascularized malignant tissue is distinguished easier from normal urothelial tissue (Figure 1c). Studies demonstrated a higher sensitivity for NBI compared to classic white-light cystoscopy, but NBI is still in the developing phase and more research is needed [12-14]. As seen with PDD, the specificity of NBI is also negatively influenced by factors, like prior use of Bacillus-Calmette Guerin (BCG), bleeding during the procedure and the presence of inflammation.

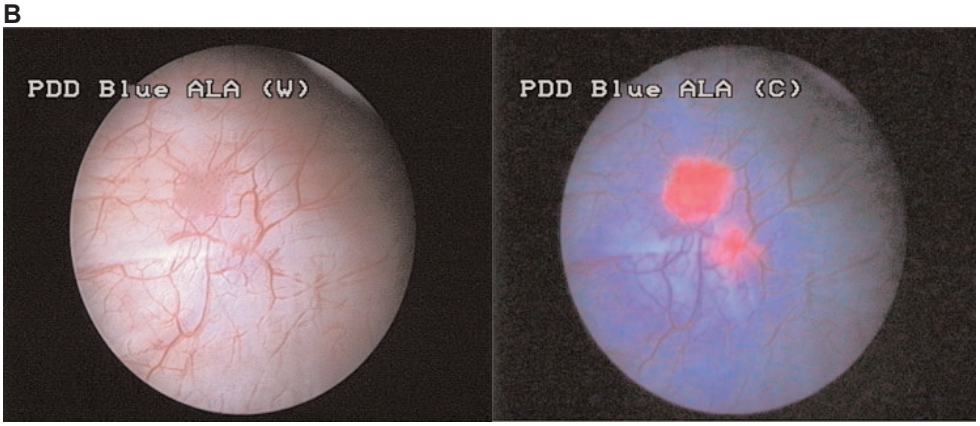
Figure 1

A



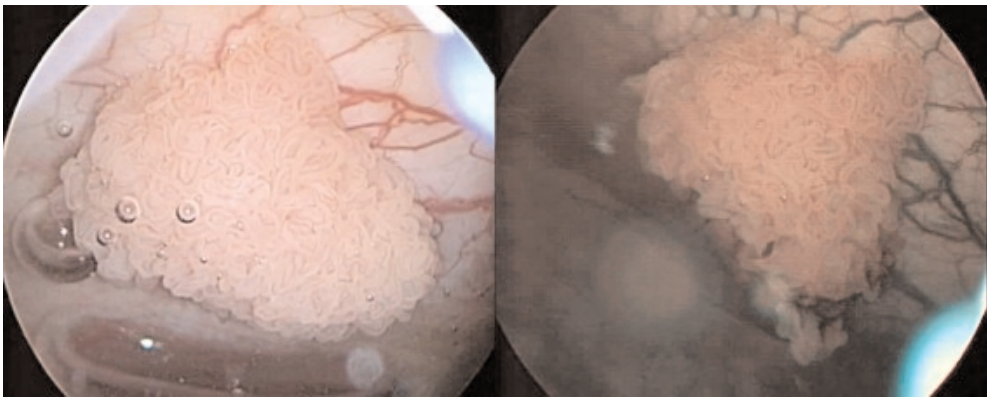
Endoscopic investigation of the bladder using a cystoscope.
From: Mayo Foundation for Medical Education and Research (MFMER). All rights reserved.

B



Photodynamic diagnosis: Detection of a papillary lesion with white light on the left and PDD on the right.
From: www.robourology.co.uk

C



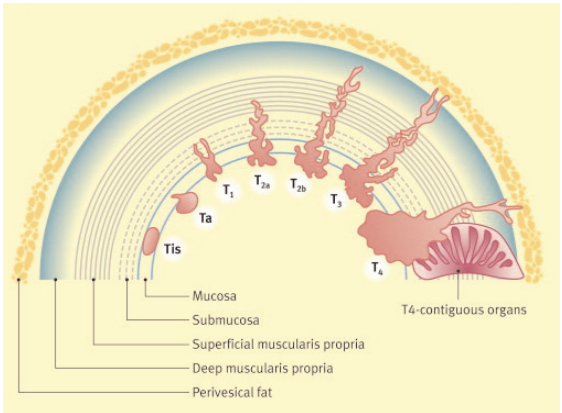
Narrow band imaging: Detection of a papillary bladder tumor with white light on the left and narrow band imaging on the right.
From: E. Cauwberg et al. Eur. Urol. 56, (2009) 287-297

When a suspicious lesion is detected in the bladder, either a biopsy is taken or a diagnostic trans-urethral resection (TUR) of the lesion is performed at a second visit. The treatment strategy following TUR is mainly determined by the histopathological outcome of the resected specimen. Residual disease and tumor recurrence are associated with incomplete TUR and therefore, it is recommended that the resected specimen should contain the deepest growing parts of the lesion to assess tumor invasion and to select the appropriate treatment.
Ultrasound or computed tomography (CT) urography can be used to visualize the upper urinary tract in case of hematuria or positive urine cytology in the absence of a cystoscopically evident bladder lesion.

1.3 Pathological staging and grading

Histopathological staging of bladder tumors is performed according to the 2009 TNM-classification system approved by the Union International Contre le Cancer. In this system, T-stage describes the extent of tumor invasion into the bladder wall, N-stage describes the involvement of regional lymph nodes and M-stage describes the presence of distant metastasis (Figure 2).

Figure 2



Histopathological staging of bladder cancer according to the 2009 TNM classification system.

Tis : Carcinoma in situ,

Ta : non-invasive papillary carcinoma,

T1 : tumor invades subepithelial lamina propria,

T2a: tumor invades superficial muscle (inner half),

T2b: tumor invades deep muscle (outer half),

T3 : tumor invades perivesical fat,

T4 : tumor invades surrounding structures (prostate, uterus, vagina, pelvic wall, abdominal wall)

From: Turo et al. Medicine Volume 40, Issue 1, January 2012, Pages 14-19

Up to 2004, bladder tumors were graded according to the 1973 World Health Organization (WHO) classification system (Figure 3a). This system classifies tumors according to the grade of differentiation of urothelial tumor cells (grade 1 to 3). The psychological and financial burden of having "cancer" in a subset of patients with low-grade tumors, which have a very low risk of progression, and the high interobserver variability between pathologists lead to the development of the new WHO grading system in 2004.
The new system distinguishes flat lesions (hyperplasia, reactive atypia, dysplasia and CIS) and papillary lesions (benign urothelial papilloma, papillary lesion of low malignant potential (PUNLMP), low- and high grade papillary urothelial carcinoma (Figure 3a, 3b)).
Although the WHO 2004 grading system shows somewhat less interobserver variability, there is still debate on the prognostic value of one grading system over the other in the absence of large prospective trials. As most studies are performed with the WHO 1973 grading system and globally accepted guidelines are based on this grading system, we used the 1973 system in our studies.

Figure 3a

1973 WHO grading
Urothelial papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated

2004 WHO grading
Flat lesions
Hyperplasia (flat lesion without atypia or papillary aspects)
Reactive atypia (flat lesion with atypia)
Atypia of unknown significance
Urothelial dysplasia
Urothelial CIS
Papillary lesions
Urothelial papilloma (completely benign lesion)
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma

Figure 3b

The WHO 2004 system compared to the WHO 1973 system for tumor grade.
From: Maclennan et al. Eur Urol 2007 Apr;51(4):889-98

1.4 Treatment

BC is divided into non-muscle invasive (NMI; Tis, Ta, T1) or muscle-invasive (MI; T2, T3, T4) disease (Figure 2). Almost 80% of the BC patients present with NMI tumors and the prognosis is good with a five-year survival of 80-90%. Tumors are removed by TUR, but 70% of the patients will have at least one recurrence within five years [3]. Approximately 10-20% of these patients will have progression to MI disease [15, 16]. After diagnostic TUR, NMIBC patients are divided into subgroups according to the probability of recurrence and progression (Figure 4). This subdivision is used to select the appropriate adjuvant treatment. Patients at a low risk of recurrence or progression should receive one immediate intravesical instillation of chemotherapy (epirubicine, doxorubicin or Mitomycin C) within 24 hours following TUR. Patients with an intermediate or high risk of recurrence and progression should receive one immediate intravesical instillation followed by BCG instillations for at least one year or should receive a radical cystectomy if intravesical treatment fails (EAU clinical guidelines).

Patients with MIBC are primarily treated by radical cystectomy and lymph node dissection. In case of non-resectable tumors, palliative cystectomy or palliative radiotherapy can be performed. Depending on the patient performance score and comorbidity (neo)adjuvant systemic chemotherapy can be offered. Despite these treatment options, the 5-year survival rate of MIBC patients is less than 50%.

1.5 Surveillance in non-muscle invasive bladder cancer

Currently, frequency of follow-up cystoscopies is determined by the risk of recurrence and progression according to the European Association of Urology (EAU) (Figure 4).

The most important factor predicting recurrence and progression is outcome at first cystoscopy following TUR. Therefore, cystoscopy is performed at 3 months after TUR in all NMIBC patients. Low-risk tumors are stage Ta, grade 1-2, small tumor size, solitary lesions and the absence of CIS. Since these patients mostly have recurrences of low stage and grade with a low risk of progression, cystoscopy is recommended at 3 months, and if negative at 9 months and then yearly for 5 years. A high-risk profile includes tumors of stage T1, grade 3, large in size, multiple lesions and the presence of CIS. These patients should receive a cystoscopy and urine cytology at 3 months, if negative a cystoscopy should be performed every 3 months for 2 years, every 6 months thereafter up to 5 years and then yearly, including yearly CT-urography of the upper urinary tract. According to the European guidelines, patients with an intermediate-risk should receive an in-between follow-up schedule with cystoscopies and urine cytology adapted to patient individual risk factors. In all groups, patients restart their follow-up schedule when a recurrence is detected.

1.6 Urinary biomarkers for the surveillance of non-muscle invasive bladder cancer

The high recurrence rate and progression to MI disease in 10-20% of NMIBC patients necessitates lifelong frequent cystoscopies and urine cytology. Although urine cytology and cystoscopy are currently the gold standard, there are still some limitations. Urine cytology has a low sensitivity for the detection of low-grade bladder tumors (7-46%) [17, 18]. Flat carcinoma of the bladder, i.e. carcinoma in situ, can be missed easily by cystoscopy and assessment of stage and grade of the tumor is very difficult, even when cystoscopy is performed by an experienced urologist. Additionally, upper urinary tract tumors cannot be detected. Cystoscopy is a costly and invasive procedure causing pain and discomfort in one-third of the patients [19].

A urine-based marker to predict the presence of a recurrence could therefore reduce the number of cystoscopies (and costs), improve patient quality of life, improve prognosis by early detection and create possibilities for new treatment options.

Figure 4

Factor	Recurrence	Progression
Number of tumors		
Single	0	0
2-7	3	3
>8	6	3
Tumor diameter		
<3 cm	0	0
≥3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤1 recurrence/year	2	2
>1 recurrence/year	4	2
T Category		
Ta	0	0
T1	1	4
Concurrent CIS		
No	0	0
Yes	1	6
Grade (WHO 1973)		
G1	0	0
G2	1	0
G3	2	5
Total score	0-17	0-23

Patient weight scores for recurrence and progression according to clinical and pathological factors. CIS = Carcinoma in situ, WHO = World health organization
From: EAU guidelines, Sylvester et al. Eur Urol 49(2006): 466-477

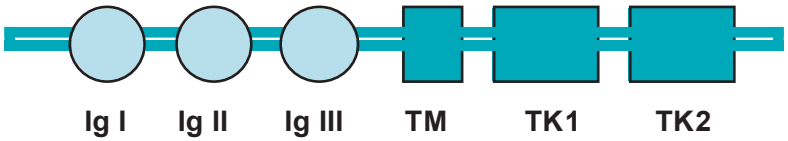
Recurrence score	Probability of recurrence at 1 year (95% CI)	Probability of recurrence at 5 years (95% CI)
0	15% (10,19)	31% (24,37)
1-4	24% (21,26)	46% (42,49)
5-9	38% (35,41)	62% (58,65)
10-17	61% (55,67)	78% (73,84)
Progression score	**Probability of progression at 1 year (95% CI)**	**Probability of progression at 5 years (95% CI)**
0	0.2% (0,0.7)	0.8% (0,1.7)
2-6	1.0% (0.4,1.6)	6% (5,8)
7-13	5% (4,7)	17% (14,20)

Recurrence and progression probability according to the patient weight score
From: EAU guidelines, Sylvester et al. Eur Urol 49(2006): 466-477

There are some general requirements that should be considered in the development of a urine-based marker. A urine-based assay should be easy to perform (also in non-academic centers) with a high diagnostic accuracy (high sensitivity and specificity), low costs and should be reproducible. At this moment, only two commercially available tests, which have been approved by the United States Food and Drug administration (FDA) seem suitable for surveillance of patients with NMIBC. ImmunoCyt has a higher sensitivity (60%) than cytology (7-46%) for the detection of low-grade tumors, but has a lower specificity, requires trained personnel, has high costs and a lot of material is needed to perform the assay.
The UroVysion® fluorescence in situ hybridization (FISH) test has a higher sensitivity (66-70%) than cytology for the detection of high-grade tumors, but is laborious, costly and a lot of material is needed to perform the assay [20]. Promising biomarkers, which are under investigation and used in this thesis will be described in the following section.
High-risk BC patients are treated with multiple BCG instillations, which could cause severe side effects, like urinary tract infections, frequency (frequent urination), hematuria and fever, affecting full compliance of treatment. After multiple sessions of BCG over a period of weeks patients receive a cystoscopy and depending on the outcome, they either continue maintenance BCG or receive a cystectomy. Although it would be beneficial to have a biomarker predicting which patients will respond to BCG treatment, currently no clinically applicable marker has been developed. Small studies have reported detection of inflammatory markers in urine during BCG treatment, like dendritic cells and cytokines. Other interesting markers that can be used to select patients prior to BCG treatment are detection of cytokine gene polymorphisms and gene profiling with microarray technology. The possibilities of theses markers at different phases during treatment will be discussed in this thesis.

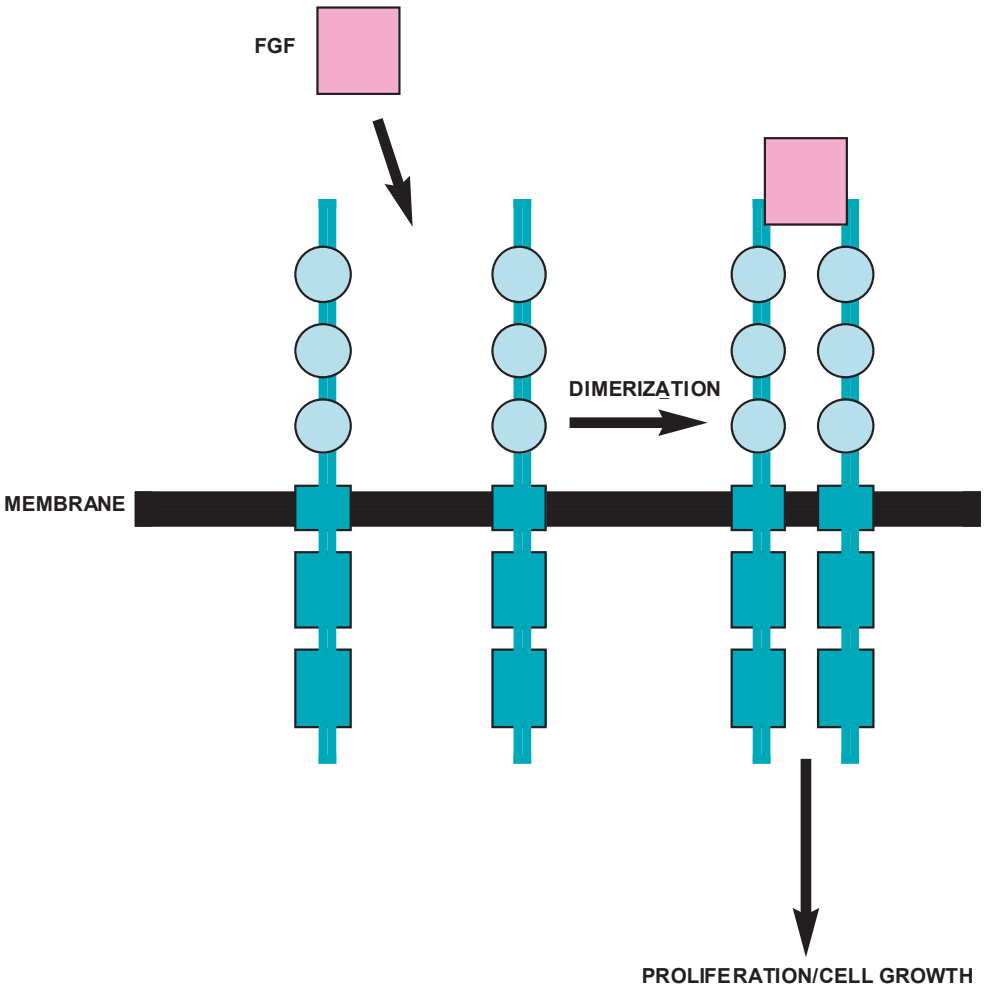
Fibroblast growth factor receptor-3 gene mutations
The fibroblast growth factor receptor (*FGFR*) family consists of four members (*FGFR 1-4*). The receptors have a common structure consisting of a ligand binding extracellular domain with three immunoglobin (Ig)-like domains, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain (Figure 5a). Binding of a ligand (fibroblast growth factor) causes dimerization of two receptors and this activates the tyrosine kinase domain regulating intracellular signaling. The *FGFR3* gene is located on chromosome 4p16.3. There are multiple isoforms of *FGFR3* that are expressed in different tissues of the body, e.g. bone *FGFR3*c and epithelial cells *FGFR3*.
Cappellen et al. were the first to identify mutations of the *FGFR3* gene in bladder tumors [21]. Previously, mutations in *FGFR3* were known to be associated with skeletal disorders, such as hypochondroplasia, achondroplasia, severe achondroplasia with developmental delay and acanthosis, nigricans (SADDAN) and thanatophoric dysplasia. In these diseases, activating point mutations of *FGFR3* lead to premature differentiation of chondrocytes in the growth plate causing dwarfism [22-25]. Oncogenic properties of *FGFR3* were identified in multiple myeloma (MM) where 20-25% of the tumor cells contained a chromosomal translocation t(4;14)p16.3;q32.3) that was associated with overexpression of *FGFR3* [26, 27]. This lead to the question whether *FGFR3* played a role in epithelial carcinomas and Cappellen et al. demonstrated the presence of *FGFR3* mutations in 35% of the investigated bladder tumors [21]. In the following years, 11 missense mutations leading to ligand-independent activation of the receptor (Figure 5b), have been identified. Interestingly, it was found that these mutations were associated with tumors of low stage and grade [28, 29]. Subsequently, studies demonstrated that *FGFR3* mutations were associated with a low frequency of chromosomal alterations and a low risk of progression to MI disease [30-33].

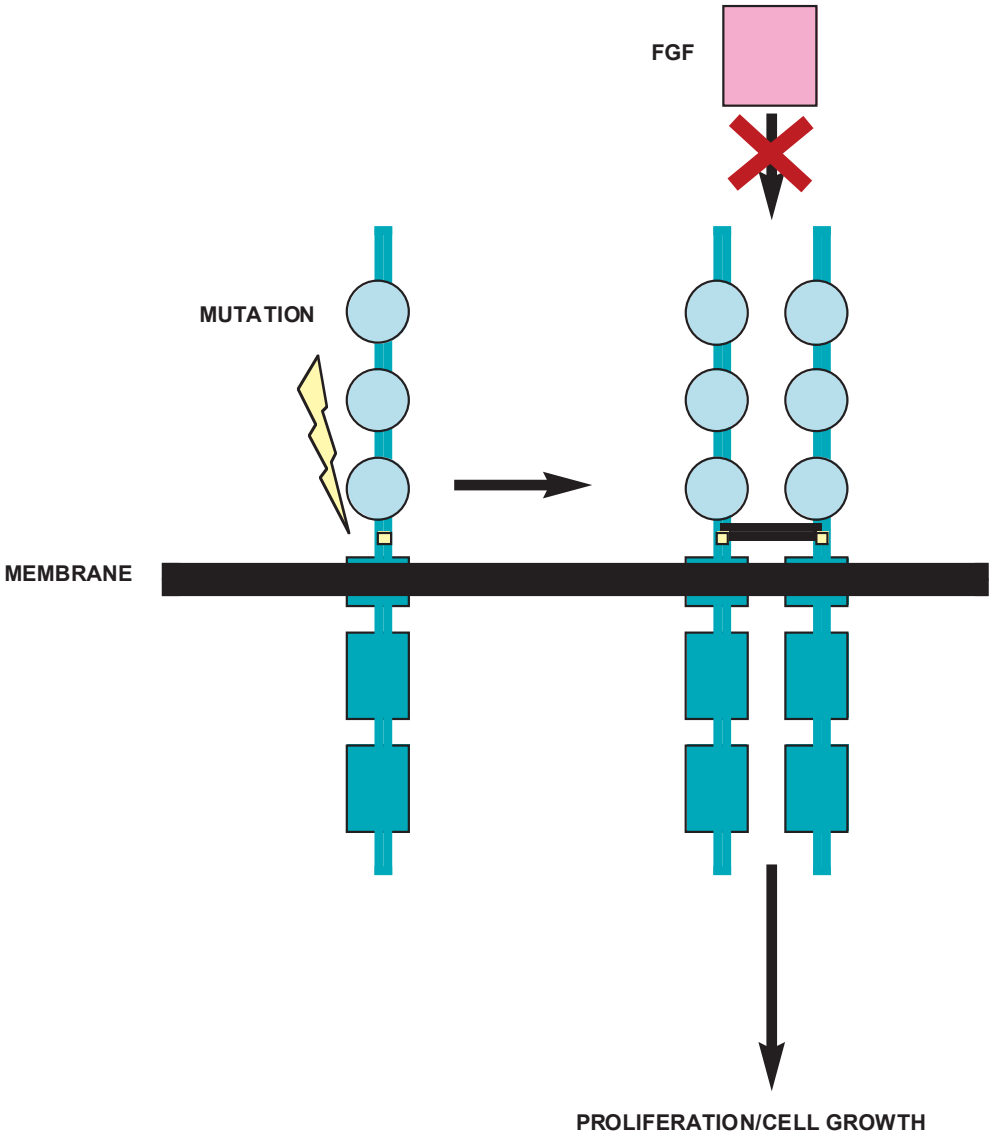
Figure 5a



Structure of the FGFR3 protein. Extracellular domains include three immunoglobin-like domains (Ig), a transmembrane domain (TM) and two intracellular kinase domains (TK).

Figure 5b





Normal activation of the FGFR3 receptor requires binding of FGF, leading to intracellular signaling. The most frequent point mutations lead to a change of an amino acid to a cysteine residue. A cysteine from one receptor forms a stable disulfide bond with another receptor, resulting in ligant-independent dimerization and continuous signaling.

Controversial findings have been found on the recurrence rate of *FGFR3* mutant tumors, but most studies show the same recurrence rate in wild-type and mutant tumors. A large study by Kompier et al. demonstrated that patients with a primary *FGFR3* mutant tumor mostly had *FGFR3* mutant recurrences (81%) and these recurrences were of low stage and grade [3]. Together, these findings combined with the absence of mutations in urothelium and urine from patients without bladder cancer could suggest that a reduced cystoscopy frequency alternated with urine-based *FGFR3* analysis is worthwhile exploring and will be investigated in this thesis [34].

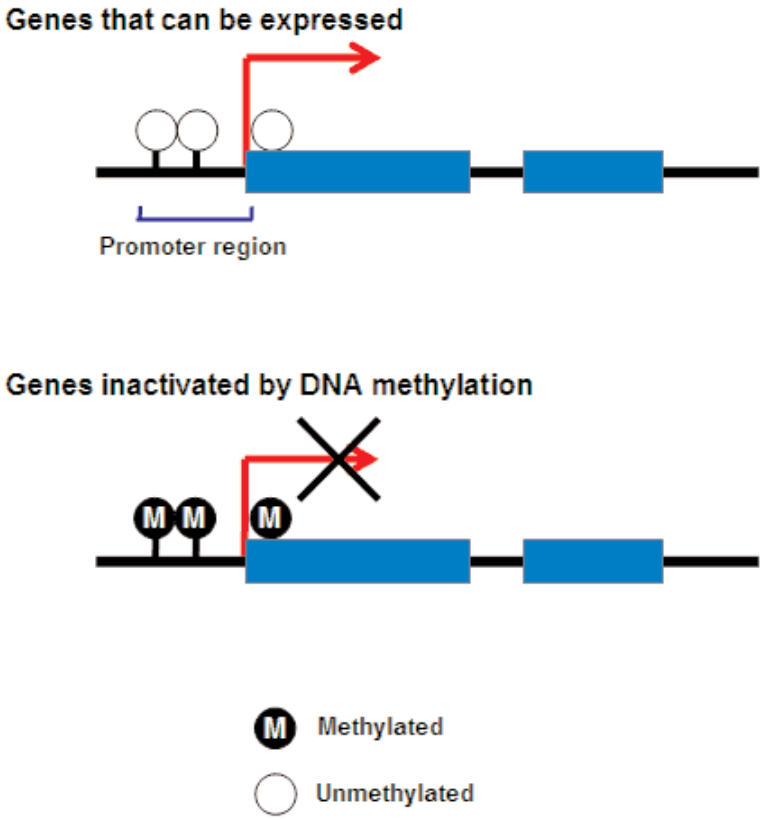
Detection of loss of heterozygosity

Microsatellites are repetitive sequences that are frequent in the genome. The repeated sequence can be a di-, tri or tetranucleotide. The microsatellites are highly polymorphic, which makes it possible to distinguish between two copies of a chromosome. Some tumors may lose part of their chromosomal regions including microsatellites, which can be detected by microsatellite analysis (MA) (PCR reaction). This phenomenon is referred to as loss of heterozygosity (LOH). The most commonly found genetic alteration in bladder tumors is LOH of chromosome 9p and 9q [35]. Less frequently, chromosomes 4p, 8p, 11p and 17p display LOH [36]. In 1997, Steiner et al. first described the use of MA for the detection of bladder cancer recurrences in urine. Soon thereafter, many studies investigated whether urine analysis for the detection of recurrences was feasible. Sensitivity for recurrent detection ranged from 72-97% and specificity from 80-100%, specifically having a high sensitivity for the detection of low-grade tumors [35]. The largest prospective study on low grade NMIBC by van der Aa et al. demonstrated a sensitivity of 58%, specificity of 73% for the detection of concurrent recurrences and a sensitivity of 83% including prediction of future recurrences [37]. Although these findings seem promising, MA is a laborious technique, with the need of blood samples as a reference. Recent developmental efforts have suggested the use of standardized markers to reduce the costs and laborious efforts [38].

Methylation markers

Methylation is an epigenetic change of DNA, meaning that the DNA sequence remains unchanged. DNA methylation is involved in different processes like genomic imprinting, embryonic development and X-chromosome inactivation [39, 40]. DNA methyltransferases (DNMT) are enzymes that methylate DNA and this typically occurs at CpG dinucleotide clusters, referred to as CpG islands. CpG islands are mainly located at promoters of genes and are normally unmethylated to allow binding of transcription factors, hereby initiating gene transcription (Figure 6). Hypermethylation of CpG islands leads to silencing of genes and is a frequent finding in different cancer types. Hypermethylation in bladder cancer was first described in 1995 by Gonzalez-Zulueta et al. and subsequent studies identified many genes to be aberrantly methylated in bladder cancer [41]. This lead to the hypothesis that detection of hypermethylated genes in voided urine could be used to identify recurrences in BC patients under surveillance. Multiple hypermethylated genes have been identified as possible targets during surveillance and in some cases even outperformed urine cytology. The methylation status of a tumor has been associated with age, gender, tumor location, stage, recurrence rate and progression. There are some important factors to consider when identifying methylation markers. Since methylation increases with age (in all individuals) this emphasizes the importance of age-matched controls [42]. Secondly, mostly primary tumors are used to select methylated genes and since these tumors are generally larger in size than recurrences, they are easier to detect than recurrences. Additionally, the use of MI tumors for the identification of genes for surveillance of NMIBC patients should be avoided, since methylation increases with stage and grade. In summary, detection of hypermethylated genes in voided urine of NMIBC patients under surveillance seems promising and development of a methylation detection assay will be discussed in this thesis.

Figure 6



CpG islands located in the promoter of a gene are unmethylated in the normal situation, enabling transcription factors to bind to the promoter and to initiate transcription. DNA-methyltransferases methylate CpG islands, thereby hindering the binding of transcription factors, leading to silencing of the tumor suppressor gene.

From: website of the National Cancer Center Research Institute

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Scope of the Thesis

The first part of the thesis is an introduction on how bladder cancer is diagnosed and treated. The surveillance protocol is outlined and the encountered difficulties associated with this protocol are discussed. An outline is given on the different biomarkers, like FGFR3 mutation detection, methylation detection and markers predicting a response to BCG treatment, which have been investigated in this thesis.

The second part discusses the possibilities of FGFR3 mutation detection as a diagnostic tool. As previous research demonstrates the high prevalence of *FGFR3* mutations in NMIBC, we first focus on the potential of *FGFR3* mutation detection on voided urine as a diagnostic tool in patients with NMIBC **(Chapter 2)**. To this end, we optimize the *FGFR3* mutation detection assay on urine by adding probes for recently discovered mutations. We then determine the sensitivity of the *FGFR3* mutation assay for the detection of concomitant and future recurrences in multiple urine samples from low-grade NMIBC patients. Additionally, we investigate the predictive value and hazard risk associated with having an *FGFR3* mutation in a urine sample.

Next, we investigate whether molecular changes detected in tumors of young patients with NMIBC are comparable to molecular changes found in elderly patients **(Chapter 3)**. A case study will be performed to gain more insight in the molecular pathogenesis of bladder tumors in young patients. We explore the presence of chromosomal aberrations (LOH) and *FGFR3* mutations in the primary tumor and recurrences of a 26-year old patient. Paraffin-embedded samples of these tumors are used to determine the expression levels of the cell cycle regulators P53 and Ki-67. Findings are compared to genetic changes in tumors of older patients. In addition, we determine the feasibility of a urine-based follow-up in this patient.

In the following chapter, we optimize the *FGFR3* mutation assay for the detection of recurrences in urine **(Chapter 4)**. To this end, we first determine whether the sensitivity for the detection of *FGFR3* mutations in urine is dependent on tumor size and the time point of urine collection. We hypothesize that urine collected in the morning is concentrated overnight and thus contains more tumor cells which could improve the sensitivity of the assay. Urine samples are collected 6 days prior to transurethral resection of the tumor and analyzed by *FGFR3* mutation analysis and we determine the sensitivity of the assay to predict a recurrence. Moreover, we determine whether morning urine contains more tumor cells than urine collected throughout the day. Lastly, we attempt to increase the sensitivity of the assay by virtually pooling urine samples collected over 24 hours.

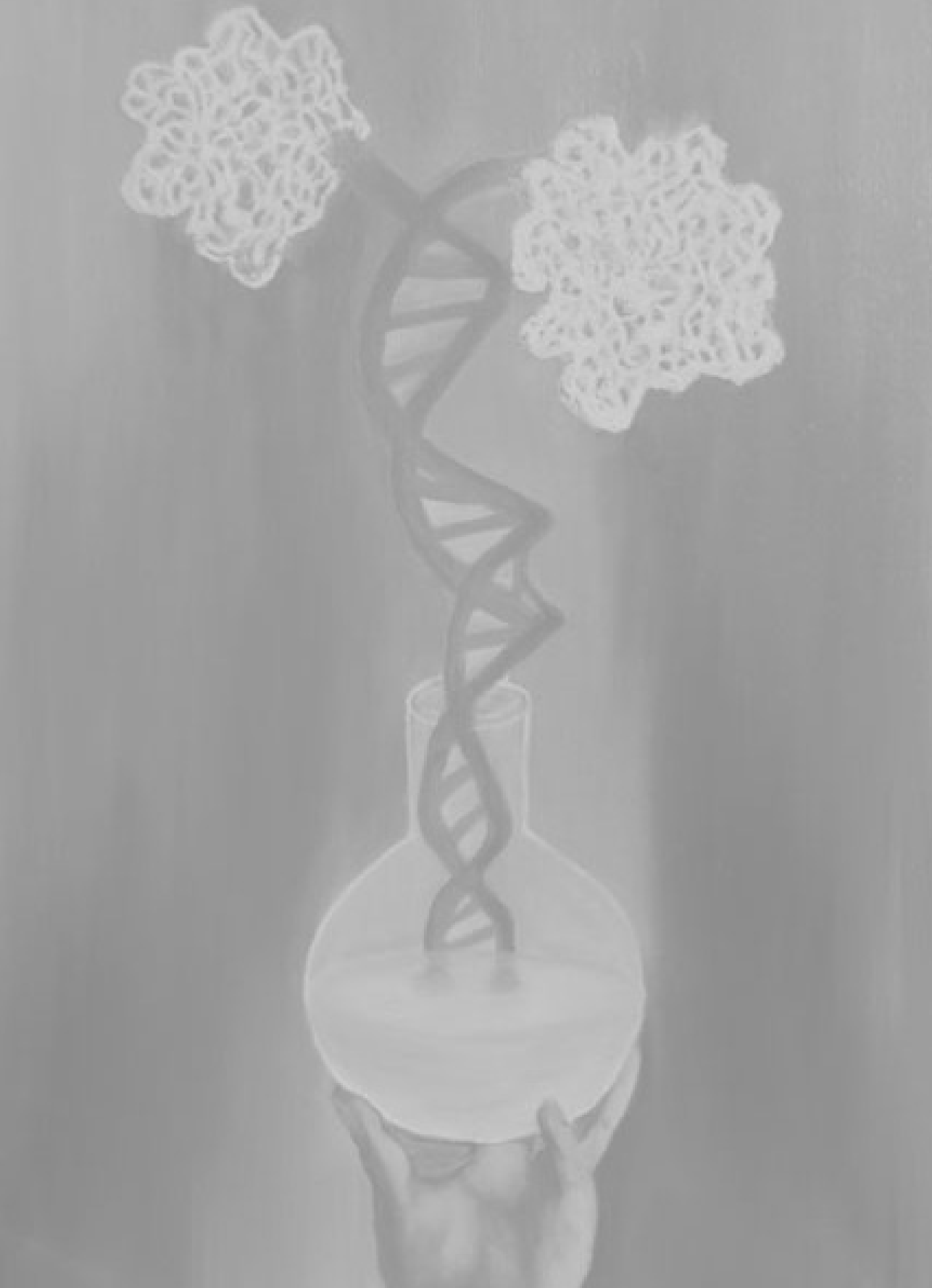
There is still debate on the prevalence of *FGFR3* mutations in prostate cancer and we determine the prevalence of these mutations in a large series of primary and locally advanced prostate tumors (n=132) **(Chapter 5)**. We investigate whether the presence of an *FGFR3* mutation is associated with a good prognosis as previously found in bladder tumors. Additionally, we investigate the prevalence of *FGFR3* mutations in prostate cancer patients with coexistent tumors in other tissues. 28 Patients had a coexistent primary tumor (bladder, skin, renal cell, pancreas, gastric, colon, hepatic and lung carcinoma) and the tumors will be analyzed by *FGFR3* mutation analysis.

The next part focuses on whether the surveillance of patients with low-grade NMI bladder cancer can be improved by using different urine-based biomarkers. Previous research has shown the potential of methylation detection in urine samples and first we will design a urine-based methylation assay for the detection of recurrences in NMIBC patients **(Chapter 6)**. Genes frequently methylated in recurrences of NMIBC patients are selected to develop a BC-specific methylation assay. A gene panel with the highest predictive value will be selected from the initial assay, tested and validated on urine samples from patients with a confirmed recurrence, urine from recurrence-free patients andurine from patients without BC.

Finally, a model based on the methylation status of the gene panel will be developed and validated to predict the probability of having a recurrence.

Next, we determine the sensitivity of a single urine assay (methylation detection, FGFR3/PIK3CA/RAS mutation analysis and MA analysis) and whether a combination of these assays can improve the detection of recurrent bladder tumors **(Chapter 7)**. This is done on retrospectively collected urine samples from NMIBC (G1/G2) patients (n=136) and compared to the gold standard urine cytology. Patients with at least three follow-up urine samples after the primary tumor are included. FGFR3, PIK3CA and RAS mutation analysis, MA and methylation analysis (MS-MLPA) are performed on tissue of the primary tumor and follow-up urine DNA samples. We determine whether a combination of molecular markers increases the percentage of patients eligible for urine-based follow-up. The sensitivity and specificity of the marker combinations for the detection of recurrences are determined and compared to urine cytology.

The final part discusses the potential of different urinary markers to predict the response to BCG treatment in patients with high-risk bladder cancer **(Chapter 8)**. Current treatment for patients with high-grade NMIBC consists of intravesical BCG instillations. Up to now, none of the investigated markers have been able to predict whether patients will respond to treatment with BCG instillations. Here, we perform a systematic review from 1996-2010 on the available literature on markers predicting BCG response, discuss the key issues concerning identification of predictive markers, and provide recommendations for further research studies.



PART II

MUTATIONS IN THE FGFR3 GENE AS A DIAGNOSTIC TOOL

Based on:

Chapter 2

Fibroblast growth factor receptor 3 mutation analysis on voided urine for surveillance of patients with low-grade non-muscle-invasive bladder cancer

Chapter 3

In-depth investigation of the molecular pathogenesis of bladder cancer in a unique 26-year old patient with extensive multifocal disease: a case report

Chapter 4

Optimization of non-muscle invasive bladder cancer recurrence detection using a urine based FGFR3 mutation assay

Chapter 5

No evidence of FGFR3 mutations in prostate cancer

CHAPTER 2

Fibroblast growth factor receptor 3 mutation analysis on voided urine for surveillance of patients with low-grade non-muscle-invasive bladder cancer

Clin Cancer Res. 2010 Jun 1;16(11):3011-8

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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Fibroblast Growth Factor Receptor 3 Mutation Analysis on Voided Urine for Surveillance of Patients with Low-Grade Non-Muscle–Invasive Bladder Cancer

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Abstract

Purpose: Mutations in the *fibroblast growth factor receptor 3* (*FGFR3*) have been found in 70% of the low-grade non-muscle–invasive bladder cancer (NMI-BC) tumors. We aim to determine the potential of *FGFR3* mutation analysis on voided urine to detect recurrences during surveillance of patients with low-grade NMI-BC.

Experimental Design: *FGFR3* mutation status of the study inclusion tumor was determined from 200 low-grade NMI-BC patients. Patients with an *FGFR3*-mutant inclusion tumor were selected for analysis and monitored by cystoscopy, and voided urine samples were collected. *FGFR3* mutation analysis was done on 463 prospectively collected urines. Sensitivity and predictive value of the assay were determined for detection of concomitant recurrences. Longitudinal and Cox time-to-event analyses were done to determine the predictive value for detection of future recurrences.

Results: Median follow-up was 3.5 years. The sensitivity of the assay for detection of concomitant recurrences was 26 of 45 (58%). Of the 105 positive urine samples, 85 (81%) were associated with a concomitant or a future recurrence. An *FGFR3*-positive urine was associated with a 3.8-fold ($P < 0.0001$) higher risk of having a recurrence in the Cox analysis. In contrast, only 41 of 358 (11%) *FGFR3*-negative urine samples were associated with a recurrence. Positive predictive value increased from 25% to 90% in patients having consecutive *FGFR3*-positive urine tests.

Conclusions: *FGFR3* mutation analysis on voided urine is a simple and noninvasive diagnostic method for detection of recurrences during surveillance of patients presenting with a low-grade *FGFR3*-mutant NMI-BC tumor. *Clin Cancer Res; 16(11); 3011–8. ©2010 AACR.*

Bladder cancer (BC) is the fifth most common malignancy in the world and can be divided into non-muscle–invasive BC (NMI-BC) and muscle-invasive BC tumors (MI-BC; ref. 1). Almost 80% of the NMI-BC patients will present with a G1 or G2, low-grade NMI tumor, making this a considerable burden on the urological practice. The prognosis of low-grade NMI-BC patients is good with a 5-year survival of 80% to 90% (2, 3). NMI bladder tumors are treated by transurethral resection, but 70% of the patients will develop at least one recurrence within 5 years and 10% to 20% will progress to MI disease. Currently, the surveillance protocol consists of cystoscopy every 3 to 6 months for the first 2 years, followed by less frequent observations when a patient stays recurrence-free (4). The frequent transurethral resections and follow-up cystoscopies make BC from diagnosis to death the most costly cancer in the world (5–8). The life-long follow-up of patients by invasive cystoscopy and associated high costs emphasize the need for a urinary biomarker, which can be used in a noninvasive assay for the detection of recurrences and to stratify patients with a high-risk profile, hereby creating the possibility to reduce the number of cystoscopies and thus improving quality of life.

About 70% of low-grade NMI tumors have a mutation in the *fibroblast growth factor receptor 3* (*FGFR3*) gene (9–12). In addition, it was shown that patients with an *FGFR3* mutation have a good prognosis (13–17). Because low-grade NMI-BC patients comprise the largest group in the BC urological practice, *FGFR3* mutation analysis could serve as a biomarker for the detection of recurrences during follow-up and aid in identifying patients with a low risk of progression. Two studies using single-strand polymorphism analysis for *FGFR3* mutations showed that it is possible to detect recurrent tumors in urine (12, 18).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-09-3013

Translational Relevance

The high recurrence rate of low-grade non-muscle–invasive bladder tumors necessitates life-long frequent cystoscopic monitoring of patients, and this affects patient quality of life. Fibroblast growth factor receptor 3 (*FGFR3*) mutations are found in 70% of the low-grade tumors and associated with a good prognosis. Surveillance with *FGFR3* mutation detection on voided urine could ultimately reduce the number of cystoscopies and improve patient quality of life. We investigated whether *FGFR3* mutation analysis could detect concomitant recurrences and predict future recurrences. We determined the recurrence risk associated with an *FGFR3*-positive urine and the predictive value of consecutive *FGFR3*-positive urines in time. Our findings suggest that an *FGFR3*-based and individualized surveillance protocol could be possible and having an *FGFR3*-positive urine is associated with a four times higher recurrence risk.

Because this technique is rather laborious, we developed a multiplex assay to identify the most common *FGFR3* mutations and showed its potential on urine and tissue samples (19). In the present study, we optimized this assay and validated it in a longitudinal study that determines the recurrence risk associated with *FGFR3*-positive urine samples in a large patient cohort with *FGFR3*-mutant low-grade NMI-BC.

Materials and Methods

Patients and sample collection

Patients with available urine samples were selected from the participants of the "Cost-Effectiveness of Follow-up of patients with non-muscle invasive Bladder cancer trial" (CEFuB trial; ref. 20). Patients with stage pTa or pT1 and grade 1 to 2 tumors were included at transurethral resection. Patients with a history of carcinoma *in situ* were excluded from participation. A cystoscopic examination coincided with urine collection for molecular analysis, or urine was collected 1 month following the cystoscopy due to logistic reasons. Recurrence was defined as a histologically proven tumor. Progression was defined as progression to MI disease.

Tissue samples

Tissue for DNA extraction was obtained by manual dissection from formalin-fixed, archival paraffin blocks containing tumor areas that were selected by pathologic examination of the corresponding histologic slides to contain a minimum amount of 70% tumor tissue. All tumors were reviewed by an expert pathologist (T.H.v.d.K.). Samples were first deparaffinized and DNA was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen GmbH) according to the manufacturer's protocol.

Urine samples

Freshly voided urine (10-100 mL) was collected before cystoscopy and stored at 4°C until transportation to the Department of Pathology at Erasmus Medical Center. Cells were pelleted by centrifugation for 10 minutes at 3,000 rpm (1,500 × *g*). Cell pellets were washed twice with 10 mL PBS, resuspended in 1 mL PBS, transferred to an Eppendorf vial, and collected by centrifugation for 5 minutes at 6,000 rpm (3,000 × *g*). Supernatant was discarded and the cell pellet was stored at −20°C until DNA isolation. DNA was extracted using the QiAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's protocol.

FGFR3 mutation analysis

A multiplex PCR of the three regions that contain the most frequent *FGFR3* mutations was done as described by van Oers et al. (19). These regions comprise the following codon mutations: R248C and S249C (exon 7); G372C, S373C, Y375C, G382R, and A393E (exon 10); and K652M, K652T, K652E, and K652Q (exon 15). Primers used are depicted in Supplementary Data S1. PCR was done in 15 μL containing 1 to 5 ng of genomic DNA, 1× PCR buffer (Promega), 1.5 mmol/L $MgCl_2$, 0.17 mmol/L deoxynucleotide triphosphates (Roche), 1.0 unit Taq polymerase (Promega), 5% glycerol (Fluka), 18 pmol of exon 7 primers, 7.5 pmol of exon 10 primers, and 10 pmol of exon 15 primers. Cycling conditions were as follows: 5 minutes at 95°C, 35 cycles at 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 45 seconds, followed by 10 minutes at 72°C. Total PCR product was treated with 3 units of shrimp alkaline phosphatase (Amersham Biosciences) and 2 units of exonuclease I (Amersham Biosciences) to remove excess primers and deoxynucleotide triphosphates. This was followed by a mutation analysis (ABI PRISM SNaPshot Multiplex kit, Applied Biosystems) using probes that anneal to the PCR product adjacent to the mutation site. Different lengths of polydeoxythymidylic acid tails were attached to the 5′-end to enable simultaneous detection on the sequencer. All probes are shown in Supplementary Data S2. The mutation detection reaction was done in 10 μL containing 1 μL of multiplex PCR product, 2.5 μL Ready reaction mix, 1× sequencing buffer, and probes as shown in Supplementary Data S2. Cycling conditions were 35 cycles of rapid thermal ramp (1.30) to 96°C, 96°C for 10 seconds, rapid thermal ramp (1.30) to 58.5°C, and 58.5 for 40 seconds, followed by treatment with 1 unit shrimp alkaline phosphatase. Separation was in a 30-minute run on 36-cm-long capillaries on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a mutation.

Statistical analysis

The Statistical Package for the Social Sciences 11.5 (SPSS, Inc.) was used for data analysis. Sensitivity, specificity, and predictive value were determined for every follow-up moment with a urinary *FGFR3* result with a concomitant cystoscopic examination. To take into account the fact that a

patient could have more than one urine measurement and that these are likely to be correlated within one patient, we calculated the sensitivity, specificity, and the predictive value with random-effect regression models. These models account for within-patient correlation and are suitable for the analysis of repeated measurements within one patient, as in our study.

In the longitudinal analysis, we determined whether a urine sample is followed by a recurrence within 12 months or within the total duration of the study period. We analyzed the relationship between a *FGFR3*-positive urine and the associated recurrence risk with a Cox proportional hazard model. A tumor yes/no was taken as the outcome. The time from first urine measurement to tumor detection or end of study was taken as duration of follow-up. Also in this analysis, we accounted for the fact that one patient could have more than one urine measurement. Therefore, the consecutive urine measurements were taken as a time-dependent covariate in the Cox model. The hazard ratio from the model can then be interpreted as the effect of a single positive urine sample on the hazard of a tumor, regardless of the previous samples in the same patients. Additionally, the predictive value of subsequent *FGFR3*-positive urine tests was determined by Kaplan-Meier longitudinal analysis. This was done by analysis of one or more consecutive follow-up visits (series) with either *FGFR3*-positive urines (persistent *FGFR3* positive) or *FGFR3*-negative series (persistent *FGFR3* negative), meaning, that a series could be defined as one or more consecutive *FGFR3*-positive or *FGFR3*-negative outcomes ending at (*a*) a recurrence [including upper urinary tract (UUT)], (*b*) a change in urine *FGFR3* status from positive to negative or negative to positive, or (*c*) at the end of follow-up. In case of (*a*) or (*b*), a new series would start. Curves of proportion recurrence were computed and stratified by mutational status using Kaplan-Meier analysis. The probability of recurrence development was compared using a log-rank statistic. Results were considered statistically significant at $P < 0.05$.

Results

Patient stratification

The *FGFR3* and progression status of the tumor was known for 292 patients, of whom 193 patients had an *FGFR3*-mutant tumor. *FGFR3*-mutant tumors showed progression to MI disease in 10 of 193 (5.2%) cases compared with 15 of 99 (15.2%) in *FGFR3* wild-type (WT) tumors ($P = 0.004$). This finding agrees with previous reports. Patients with an *FGFR3*-mutant tumor were then selected for analysis of urine samples for the detection of recurrences. From 134 patients with an *FGFR3*-mutant tumor, urine samples were available. Clinical and molecular characteristics of this population are shown in Table 1.

Table 1. Patient clinical and molecular characteristics ($n = 200$)

Age			
Mean	63.4		
SE	12.9		
Range	20-100		
Variable		***FGFR3* status inclusion tumor**	
		WT, *n* (%)	**Mutant, *n* (%)**
Gender			
Male		50 (33)	103 (67)
Female		16 (34)	31 (66)
Smoking			
No		12 (30)	28 (70)
Yes		42 (35)	78 (65)
Stage			
Ta		57 (33)	117 (67)
T1		9 (35)	17 (65)
Grade			
G1		29 (31)	64 (69)
G2		37 (35)	70 (65)

Performance of the *FGFR3* mutation assay

We optimized the *FGFR3* mutation assay by improving detection of the most common mutation, S249C, and adding two new mutations to the detection spectrum. We determined the sensitivity of the assay by diluting mutant tumor DNA with WT DNA. The results show that the two most common mutations, S249C and Y375C, can still be detected in a background of 40-fold normal DNA. For R248C and G372C, this was 25- and 10-fold, respectively. The results for the most common S249C mutation are shown in Supplementary Data S3. The *FGFR3* assay was subsequently carried out successfully on all 463 urine samples, and mutations in *FGFR3* could be detected in urinary-derived DNA (Fig. 1A-C). The analysis was done on urine samples from an age-matched patient cohort without BC ($n = 100$), and no mutations were detected.

Detection of concomitant recurrences

From the 134 patients included with an *FGFR3*-mutant tumor, 463 urine samples were analyzed for *FGFR3* mutations and 45 concomitant histologically proven recurrences were found. A selection of patients is shown in Fig. 2 to illustrate the possible groups that patients could be divided into (full figure available online as Supplementary Data S4). We discern four types of patients: (I) patients with positive urine samples and a recurrence (*FGFR3* mutation analysis predicts a recurrence), (II) patients with two or more *FGFR3*-positive urine samples in the absence of a recurrence (patients at risk for a recurrence), (III) patients with *FGFR3*-negative urine samples in the presence of a recurrence (*FGFR3* analysis does not predict a recurrence), and (IV) patients without

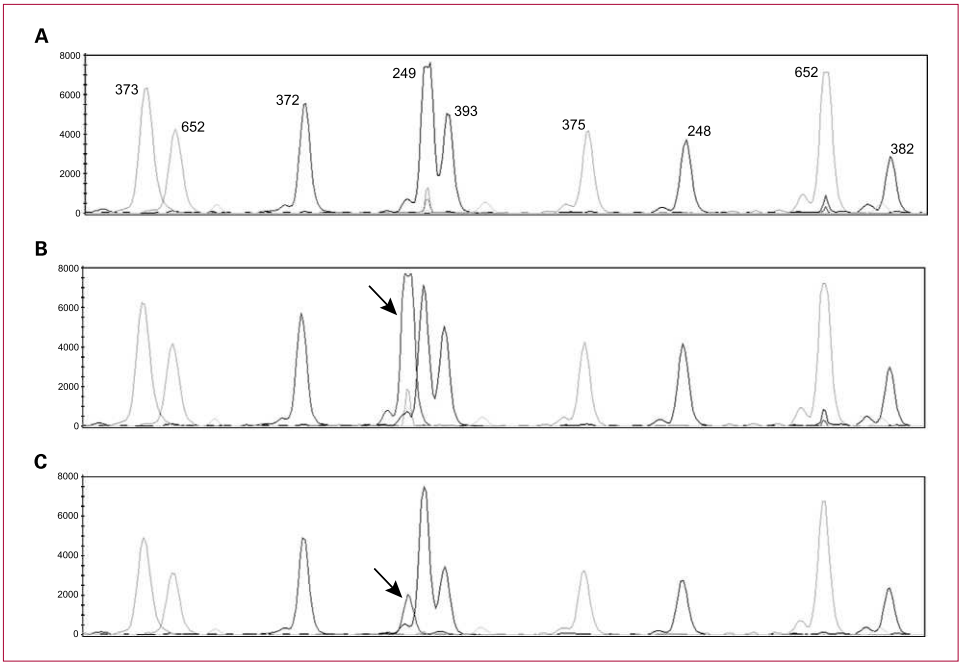

Fig. 1. *FGFR3* mutation detection possible on urinary-derived DNA. *FGFR3* mutation analysis of an *FGFR3* WT tumor (A), mutation S249C in tumor DNA (B), and urinary-derived DNA (C) from the same patient used in B. Note that overloading of S249C primer results in an off-scale signal and overflow in other color signals.

recurrences: the recurrence-free group [i.e., patients having consistently *FGFR3*-negative urine samples or a single *FGFR3*-positive urine sample ($n = 7$) within a series of negative samples]. The sensitivity for detection of a concomitant recurrence was 26 of 45 (58%), and the positive predictive value was 26 of 105 (25%). The urine assay detected three UUT recurrences that were not detected by cystoscopy, including one pT2G3. We observed that many urine tests were positive, whereas the concomitant cystoscopy did not reveal a tumor. Because *FGFR3* mutations are tumor specific and do not occur in normal tissue or urine, we argued that these represent anticipatory positive results, and therefore, we did a longitudinal analysis on the occurrence of future recurrences.

FGFR3 mutation detection for identifying patients at risk for future BC recurrences: a longitudinal analysis

Because of the anticipatory effect, we extended the time of patient follow-up so that at least a year of clinical follow-up data was available after the last urine sample had been analyzed. This increased the number of recurrences to 79 (45 concomitant and 34 future recurrences). From the 105 positive urine samples, 58 (55%) were associated with a recurrence within a year after the positive urine test and 85 of 105 (81%) with a recurrence during the total study period (Fig. 3). The remaining 20 positive urines were not followed by a recurrence in the entire follow-up period. It should be noted that eight urine samples from this group were single *FGFR3*-positive urines with a low mutant signal, depicted in Fig. 2 as patient type IV. Of the other 12 urine samples with an *FGFR3*-positive signal, 11 are from three patients representing patient type II (Fig. 2) as at risk patients because all had multiple positive urine samples. These patients had insufficient follow-up. Interestingly, multiple urine samples from patient H6 also displayed loss of heterozygosity and this patient even had macroscopic hematuria. Despite this, an intravenous pyelogram did not reveal any upper tract disease. These findings suggest that type II patients might have minimal residual disease and warrant regular examinations.

Figure 3 shows that 358 of 463 urine samples were *FGFR3* negative, of which 317 (89%) were from patients in whom no recurrence occurred. The other 41 (11%) negative samples were associated with a recurrence within 12 months after urine sample collection. However, 11 of these negative urines were within a series of multiple

positive urine tests. Therefore, these samples could reflect the absence of tumor cells in the urine. In the other 30 *FGFR3*-negative urine samples that were associated with a recurrence, only a single urine sample was available before the recurrence in 14 cases.

In a Cox time-to-event analysis, determining the hazard risk of a recurrence with an *FGFR3*-positive test, it was shown that a single positive *FGFR3* test was associated with a three times higher risk of a recurrence, regardless of the previous urine sample (hazard ratio, 3.8; $P < 0.0001$). Next, we determined the predictive value of multiple consecutive *FGFR3*-positive urine tests in time and recurrence development by Kaplan-Meier longitudinal analysis. The risk of developing a recurrence increases to 90% in patients with consecutive *FGFR3*-positive urine samples for 39 months ($P < 0.0001$, log-rank test; Fig. 4).

Clinicopathologic and molecular features of the detected and missed recurrences within 12 months following urine collection and analysis are shown in Table 2. Mostly pTaG1 and pTaG2 tumors were missed. It should be noted that one of two pT1G3 and one of two pT2G3 tumors were not detected, but in these cases, only one urine sample could be investigated before the recurrence. Three upper tract recurrences, one of which was pT2G3, were detected by the mutation analysis but not by cystoscopy. There were five patients with progression to MI disease from whom urine samples were available (Supplementary Data S4). In three of five cases, *FGFR3* urine analysis predicted the recurrence.

In summary, an *FGFR3*-positive urine is associated with a three times higher recurrence risk, and having multiple consecutive *FGFR3*-positive urine tests is strongly associated with development of a future recurrence.

Discussion

Mutations in *FGFR3* are frequent in NMI bladder and UUT tumors. This is the first study that determines the potential of urinary surveillance by *FGFR3* mutation detection on a large set of prospectively collected urine samples of low-grade NMI-BC patients presenting with an *FGFR3*-mutant tumor. In this study, 67% of the low-grade NMI tumors had a mutation in *FGFR3*, in agreement with previous studies (9 10, 12). Because these mutations are not found in urine of non-BC patients and normal urothelium, detection of an *FGFR3* mutation is a strong indicator for the presence of tumor cells in the urinary tract (21). *FGFR3* mutations could be detected in the presence of 5% tumor cells, making the assay highly sensitive for detection of the most common mutations. This is of great advantage because urine can also contain normal urothelial cells and lymphocytes. The assay is cheap with costs for consumables under $10 per sample, including DNA isolation, and can be done with only 1 ng DNA in a standard molecular diagnostics laboratory with a PCR machine and sequencer. This assay is also less labor intensive than sequence analysis.

FGFR3 analysis was carried out successfully on all urine samples in this study. The sensitivity of the *FGFR3* assay for detection of concomitant recurrences was 58%. This low concomitant sensitivity has several possible reasons. Firstly, tumor cells are not always present in urine. This is illustrated in Fig. 2, where a subset of patients (bottom

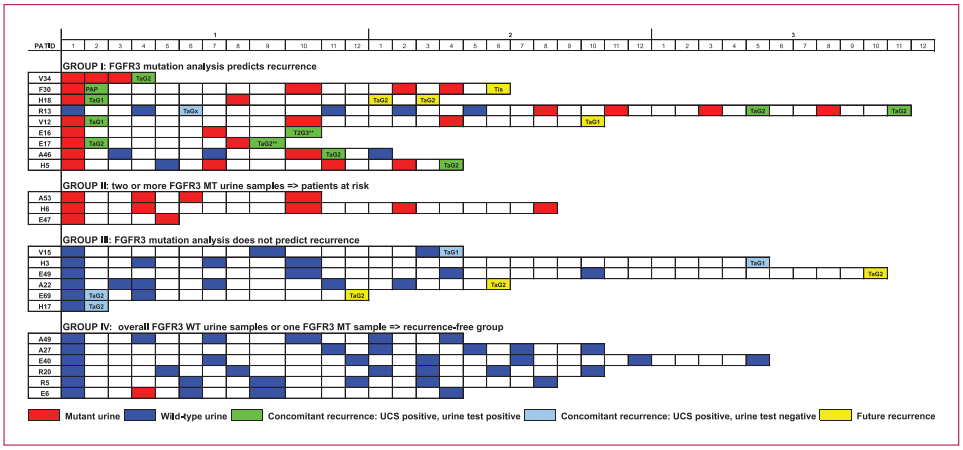

Fig. 2. *FGFR3* mutation analysis of a selection of urine samples from patients included with an *FGFR3*-mutant (MT) tumor. Patients are divided into four groups. First column displays patient identification number (PATID). Following columns depict urine samples and concomitant or future recurrences. Columns are divided into years and months. Cell colors are explained in the legend. UCS, ureterocystoscopy. **, UUT recurrence.

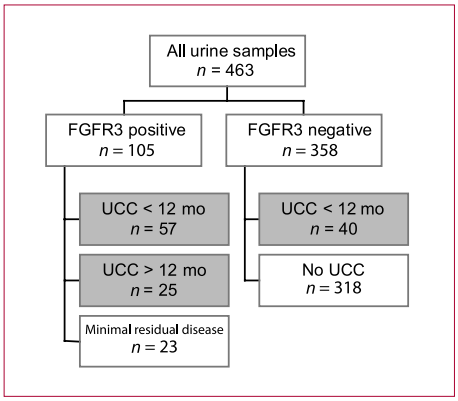

Fig. 3. *FGFR3* test results for all analyzed urines and associated future recurrences. Surveillance results of all urines from patients with an *FGFR3*-mutant inclusion tumor. Urine test results are divided into *FGFR3*-positive and *FGFR3*-negative. Analysis was based on detection of a future recurrence within 12 mo or after 12 mo of urine collection. Based on these data, the hazard ratio of a positive urine for tumor recurrence is 3.8 ($P < 0.0001$). UCC, urothelial cell carcinoma recurrence.

two, type I patients) is depicted in whom an *FGFR3*-negative urine sample is embedded between positive urines preceding a recurrence. Because this problem of urine sampling concerns all urinary tests that depend on the presence of tumor cells in the urine, this is an important issue and it would be of interest to determine the optimal time point and frequency of urine collection to improve the number of urinary tumor cells. Secondly, *FGFR3* WT recurrences are sometimes found in patients with a primary *FGFR3*-mutant tumor (22). Whether this is the reason why some recurrences were missed here is not known at present because tumor material from the recurrences was not available for *FGFR3* mutation analysis. We have previously shown that the stage and grade of these recurrences do not differ from mutant recurrences (22). Therefore, the absence of a mutation cannot be correlated with progression to a more severe phenotype. In most patients, WT recurrences are again followed by mutant recurrences. Thus, repeated urine testing should circumvent this potential problem. Thirdly, although cystoscopy is considered the golden standard, it is known that cystoscopy does not detect all tumors (22–25). Urine cytology was not routinely done in the participating centers due to the low sensitivity in tumors of low stage and grade (G1 tumors: range, 7-38%; G2 tumors: range, 18-46%), and this is considered a limitation of the study (26–29). When this work was under review, Miyake et al. published a peptide nucleic acid–mediated PCR clamping assay for detection of *FGFR3* mutations in urine samples. They were able to detect seven of nine recurrences using this assay on DNA obtained from urine samples (sensitivity, 78%). Unfortunately, the assay could not be carried out on 27% of the urine samples due to low DNA concentrations. In our study, all samples were analyzed successfully. A very interesting finding in their study is the quantitative analysis of the number of tumor cells present in urine, shown to be a prognostic indicator of tumor recurrence (30).

For the clinician, it is important to know which decision to make when confronted with an *FGFR3*-positive or *FGFR3*-negative urine test. Our data show that ~23% of the urine samples are positive for an *FGFR3* mutation in patients included with an *FGFR3*-mutant tumor (Fig. 3) and that the majority of these positive urines (81%) are associated with a recurrence. We show that a positive *FGFR3* urine is associated with a 3.8-fold higher risk to develop a recurrence, and these findings could provide a base to set up a new individualized surveillance protocol. Additionally, we show that multiple consecutive *FGFR3*-positive urines are significantly associated with a higher proportion of recurrences. In this study, patients in general had an equal number of cystoscopies, meaning, that our findings reflect a true effect of the mutation analysis and are not caused by differences in cystoscopy frequency between patients. Almost 70% of the patients presenting with a primary bladder tumor will have a mutation in *FGFR3*. We suggest that after the first transurethral resection, patients have a cystoscopy at 3-12-24 months and urinary *FGFR3* mutation analysis at 6-9-15-18-21-27-30-33 months. In the far majority of cystoscopies, following a positive test, a recurrence will be found, especially when the urologist is aware of the positive urine test result (31).

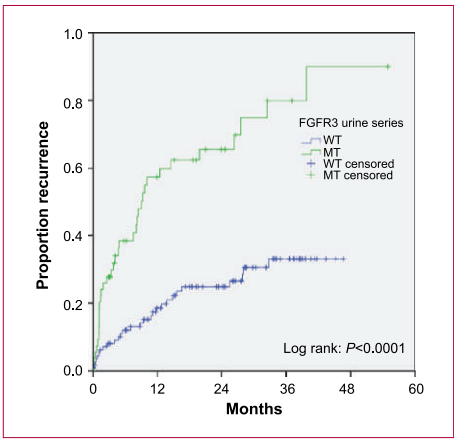

Fig. 4. Predictive value of the *FGFR3* mutation assay increases with multiple consecutive *FGFR3*-positive urine samples. Kaplan-Meier analysis of proportion recurrence for patients included with an *FGFR3*-mutant tumor. *X* axis depicts the length of time being recurrence-free with consecutive *FGFR3* urine results. *Y* axis depicts the proportion of recurrences detected.

Table 2. Fifty-seven missed and detected recurrences within 12 mo following urine analysis

	FGFR3-mutant inclusion tumors		Total
	Detected, *n* (%)	Missed, *n* (%)	
Tumor			
Papilloma	1 (33)	2 (67)	3
pTaG1	10 (55)	8 (45)	18
pTaG2	20 (68)*	9 (32)	28
pTaG3	2 (100)	0	2
pT1G3	1 (50)	1 (50)†	2
pT2G2	1 (100)	0 (0)	1
pT2G3	1 (50)*	1 (50)†	2
>pT2			
pTis	1 (100)	0	1
Total	37	21	58

*Including one UUT tumor.
†Only one urine sample tested.

Hence, we suggest that a positive test should be followed by a cystoscopy. In case of a positive urine test and a negative cystoscopy, we suggest that UUT imaging is done. Frequency of imaging should be adjusted according to the clinicopathologic features of the tumor that have been associated with an increased risk of upper tract recurrences (e.g., tumor located in trigone, multiple tumors, and high-risk tumors; refs. 32, 33). If an *FGFR3*-mutant patient presents with a negative test result during follow-up, no cystoscopy is required if urine analysis is done after 3 and 6 months following the negative test. When *FGFR3* urine analysis is implemented during surveillance, the number of cystoscopies will decrease in patients with negative urine tests and patients with positive urine tests who require more frequent cystoscopic monitoring are identified. Because ~80% of the urine tests are negative (Fig. 3), the total number of cystoscopies can be reduced substantially, hereby lowering costs and improving patient quality of life.

At this moment, the standard cystoscopic follow-up protocol is indicated in patients presenting with an *FGFR3* WT tumor because the risk of progression is higher in these patients. Detection of loss of heterozygosity by microsatellite analysis is the only evaluated molecular marker in the follow-up of patients with an *FGFR3* WT tumor (20). Although results seem promising, currently, costs are too high to implement the microsatellite analysis test in a routine clinical setting. Food and Drug Administration–approved UroVysion fluorescence *in situ* hybridization could be an alternative for detection of *FGFR3* WT high-risk recurrences but is not yet evaluated together with *FGFR3* analysis.

We have also shown that not many tumors are missed when multiple urine samples were available. Moreover, three UUT tumors were detected, which can be considered an added confidence in the detection of BC recurrences. The finding of UUT having *FGFR3* mutations agrees with a previous study by van Oers et al. (17). We also confirmed that patients with *FGFR3*-mutant tumors have a significantly lower rate of progression. NMI-BC tumors with an *FGFR3* mutation differ genetically from NMI-BC tumors that are WT in that they have fewer genomic aberrations as was indicated by loss-of-heterozygosity analyses and comparative genomic hybridization (12, 34). This suggests that the larger number of aberrations in WT tumors will affect additional cancer genes, and this may be the cause of their more aggressive behavior. In this study, *FGFR3* urine analysis predicted the recurrence in three of five cases with progression. In the other two cases where *FGFR3* analysis did not predict the recurrence, only one urine sample was collected before the tumor, in contrast to the previous three cases where multiple urine samples were available. This suggests that collection of multiple urine samples could improve detection of recurrences.

In summary, patients with a low-grade *FGFR3*-mutant NMI bladder tumor represent >50% of all patients first diagnosed with BC (15). About 70% of these patients will develop recurrences, although their progression risk is low (11, 13 14, 22). Yet they need long-term and costly follow-up by repeated cystoscopic monitoring. The data presented here suggest that three monthly urine testing alternated by, for instance, yearly cystoscopies could serve as an alternative follow-up approach for these patients. Patient stratification by *FGFR3* mutation status and such a surveillance schedule could substantially reduce the total costs, improve quality of life, and even lead to an earlier detection of recurrences, thereby preventing possible progression. Additionally, UUT recurrences are detected with this assay, which can be considered an added confidence in the diagnosis of BC. These findings are promising but preliminary due to the small number of patients and show the need for large, randomized, controlled trials to determine the feasibility of *FGFR3* mutation analysis for the detection of recurrences during surveillance of patients presenting with low-grade *FGFR3*-mutant NMI bladder tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all the urologists and pathologists who participated in this trial.

Grant Support

Koningin Wilhelmina Fonds grant 2006-3672.
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Received 11/11/2009; revised 03/22/2010; accepted 04/12/2010; published OnlineFirst 04/19/2010.

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CHAPTER 3

In-depth investigation of the molecular pathogenesis of bladder cancer in a unique 26-year old patient with extensive multifocal disease: a case report

BMC Urol. 2010 Feb 26;10:5

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CASE REPORT Open Access

In-depth investigation of the molecular pathogenesis of bladder cancer in a unique 26-year old patient with extensive multifocal disease: a case report

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Abstract

Background: The molecular characteristics and the clinical disease course of bladder cancer (BC) in young patients remain largely unresolved. All patients are monitored according to an intensive surveillance protocol and we aim to gain more insight into the molecular pathways of bladder tumors in young patients that could ultimately contribute to patient stratification, improve patient quality of life and reduce associated costs. We also determined whether a biomarker-based surveillance could be feasible.

Case Presentation: We report a unique case of a 26-year-old Caucasian male with recurrent non-muscle invasive bladder tumors occurring at a high frequency and analyzed multiple tumors (maximal pTaG2) and urine samples of this patient. Analysis included *FGFR3* mutation detection, FGFR3 and TP53 immunohistochemistry, mircosatellite analysis of markers on chromosomes 8, 9, 10, 11 and 17 and a genome wide single nucleotide polymorphism-array (SNP). All analyzed tumors contained a mutation in *FGFR3* and were associated with FGFR3 overexpression. None of the tumors showed overexpression of TP53. We found a deletion on chromosome 9 in the primary tumor and this was confirmed by the SNP-array that showed regions of loss on chromosome 9. Detection of all recurrences was possible by urinary *FGFR3* mutation analysis.

Conclusions: Our findings would suggest that the BC disease course is determined by not only a patient's age, but also by the molecular characteristics of a tumor. This young patient contained typical genetic changes found in tumors of older patients and implies a clinical disease course comparable to older patients. We demonstrate that *FGFR3* mutation analysis on voided urine is a simple non-invasive method and could serve as a feasible follow-up approach for this young patient presenting with an *FGFR3* mutant tumor.

Background

Bladder cancer (BC) is a disease of the elderly with a peak incidence in the sixth decade of life. Tumors are sporadically found under the age of 40 (1-4%) and most young patients present with tumors of low stage and grade [1-4]. Conflicting results have been found concerning the natural history and prognosis of bladder tumors in young patients. The small number of cases and the definition of "young" with age ranging from 5-45 years may be responsible for this variation [2,3,5,6]. Evidence is accumulating that there is a difference in the natural history of patients under the age of 20 and patients between 30-50 years of age. Patients <20 years mostly have tumors with a low recurrence rate, a favorable clinical outcome and few genetic alterations, while patients between 30-50 years have a disease course comparable to older patients [7].

Almost 80% of the BC patients will present with non-muscle invasive disease (NMI-BC). Treatment is by trans-urethral resection of the tumor, but almost 70% of the patients will have at least one recurrence within five years and 10-20% will progress to muscle-invasive disease. After the first tumor resection all age groups of

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patients are monitored according to an intensive surveillance protocol that includes 3-monthly cystoscopies the first two years, followed by less frequent observations if a patient stays recurrence free. The main disadvantages of the current protocol are life-long invasive and costly cystoscopic monitoring of patients causing physical discomfort and sexual dysfunction [8].

To our knowledge there are only few studies that investigated the molecular changes in bladder tumors of young patients. Identifying the molecular pathways of these tumors could define a subset of patients and redirect patient management towards a new patient friendly and individualized follow-up protocol. One of the most promising markers associated with NMI bladder tumors is the mutation status of the Fibroblast Growth Factor Receptor-3 (*FGFR3*). Mutations in *FGFR3* have been associated with BC tumors of low stage and grade and patients having a favorable prognosis [9]. We have recently shown that *FGFR3* mutation analysis on voided urine of NMI-BC patients with a mutation in *FGFR3* is a non-invasive inexpensive tool for patient surveillance (Zuiverloon *et al*. submitted). Additionally, multiple studies report on the use of microsatellite analysis (MA) for detection of loss-of-heterozygosity (LOH) as a diagnostic marker. LOH detected by MA is mainly located on chromosomes 8, 9, 10, 11, 13 and 17 and these losses have been associated with stage, grade, invasive growth, recurrent disease and progression. In the present study we analyzed multiple tumor and urine samples of a unique young patient for *FGFR3* mutation status, LOH, FGFR3 and TP53 expression and performed a genome wide single nucleotide polymorphism-array (SNP). Since this patient presented with multiple recurrences within a short time-span we determined retrospectively whether *FGFR3* mutation detection could be a feasible follow-up approach.

Case presentation

We report a unique case of a 26-year-old Caucasian male with recurrent non-muscle invasive bladder tumors occurring at a high frequency. The patient presented at first in March 2007 with macroscopic hematuria for a few weeks. He received a total of 5 trans-urethral resections of the multifocal bladder tumors within 2 years, highest stage and grade being TaG2. The first three resections included an average of 15 papillary tumors and the last two resections included 3 tumors. The patient received two direct post-operative intravesical instillations of epirubicin. Additionally, our patient initially received adjuvant intravesical immunotherapy with bacillus calmette-guérin (BCG) in 2008, but switched to mitomycin-C (MMC) in 2009 for maintenance due to complications. A CT-scan of the pelvis and abdomen demonstrated no evidence of upper urinary tract lesions, no signs of urolithiasis, nodal or distant metastases. There was no family history of bladder cancer and intoxications included a smoking status of 5.5 pack-years (one pack-year = one pack of cigarettes a day for one year ~20 cigarettes a day for one year). There was no indication of any contact with aromatic amines. The patient worked as a soldier in Bosnia in 2001 and 2003 where he was part of a recovery team driving a diesel armed-truck. Tasks of the recovery team included cleaning of remaining ammunition enforced with depleted uranium (DU) and military equipment wreckage.

Methods

Tissue samples

Tumor tissue was obtained from formalin-fixed, paraffin-embedded samples. Tumor sections were selected by pathological examination to contain a minimum amount of 80% tumor cells and sections were manually dissected from 4 μ slides. Samples were first deparaffinized and DNA was extracted using the Qiagen Dneasy blood and tissue kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol.

Urine samples

Freshly voided urine (10-100 ml) was collected prior to a cystoscopy or trans-urethral resection of the tumor and stored at 4°C until transportation to the department of Pathology at Erasmus MC, Rotterdam. Urine was spun down for 10' at 3000 rpm (1500 × g). Cell pellets were washed twice with 10 ml of Phosphate-buffered saline (PBS) and spun down for 10' at 3000 rpm. Pellets were resuspended in 1 ml of PBS, transferred to an eppendorf vial and spun down for 5' at 6000 rpm (3000 × g). Supernatant was discarded and the cell pellet was stored at -20°C until DNA isolation. DNA was extracted using the QiAamp DNA mini-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Fibroblast growth factor receptor 3 mutation analysis

The *FGFR3* mutation detection assay was performed as described previously by van Oers *et al*. [10]. In short a multiplex PCR of the three regions that contain the most frequent FGFR3 mutations (exon 7, 10 and 15) was performed. This was followed by a single nucleotide polymorphism analysis using probes that anneal to the PCR product adjacent to the mutation site. Probes were extended with a labeled dideoxynucleotide and the products were analyzed on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the label indicating the presence or absence of a mutation. Genescan Analysis Software version 3.7 (Applied Biosystems) was used for analysis of the data.

Microsatellite analysis

Microsatellite analysis was performed as described by van der Aa *et al*. [11]. Markers used for detection of LOH were: D8S1130, D81125, D8S1107, D8S1109, D8S1145, D9S1118, D9S252, D9S304, D9S299, D9S752, D9S930, G10693, D10S1225, D11S1999, D11S1981 and D17S969.

FGFR3 and TP53 immunohistochemistry

Bladder tumors were fixed in 10% buffered formaldehyde, embedded in paraffin, 4 micrometer sections were mounted on a coated glass slide (Starfrost Knittel-Glaeser D38114 Braunschweig Germany). Haematoxylin-Eosin was used as a general stain and immunohistochemistry was performed with rabbit polyclonal antibody anti-human FGFR3 (Santa Cruz Biotechnology INC) and monoclonal mouse anti-human P53 protein clone D-07 (Dakocytomation, Denmark A/S). Antigen retrieval by microwave heating in TRIS-EDTA buffer pH 9.0 was used and endogenous peroxidase was removed by 0.30% H_2O_2 in PBS/TWEEN. Pretreatment with blocking buffer PROTIFAR 0.5% in PBS/TWEEN was used prior to the overnight incubation at 4°C with the primary antibody. Visualization was done by Dako Real Envision Detection system peroxidase/DAB+ (Dakocytomation, Denmark A/S), containing anti-mouse and anti-rabbit antibodies, according to the manufacturer's protocol. Counterstaining was done with haematoxylin (Klinipath 6921 GX, Duiven, The Netherlands). Expression of FGFR3 was scored in a semi-quantitaive scoring system: 0 = all tumor cells negative, 1 = faint positivity of in some or all cells, 2 = weak but extensive positivity and 3 = strong positivity/overexpression (regardless of extent). TP53 overexression was scored if >10% of the cells stained positive. As a reference normal urothelium was analyzed for FGFR3 and TP53 protein expression. Reference sections of known high and low expressions levels of FGFR3 and TP53 were included in the staining runs.

Infinium HumanHap370CNV Genome wide SNP array

For Infinium HumanHap370CNV Genotyping BeadChip SNP array analysis we used 750 ng of patient DNA and followed the protocol as described by the manufacturer (Illumina Inc., San Diego, CA, USA). We used Illumina BeadStudio software to extract data. The Nexus CGH Plug-in for CNV Analysis from BioDiscovery (BioDiscovery Inc., El Segundo, CA, USA) was used to export Illumina CNV data to Nexus Copy Number version 4.0. Arrays were processed using the built-in Rank Segmentation algorithm.

Results and discussion

Young patients rarely present with BC and there are multiple studies that indicate a good clinical disease course where patients present with solitary tumors and a low recurrence and progression rate. Since BC patients are monitored cystoscopically according to an intensive surveillance protocol, gaining more insight into the molecular pathways of BC tumors in young patients could define a subset of patients that can be monitored less frequently, hereby improving patient quality of life and reducing associated costs.

We presented a unique case of a 26-year-old male with multiple multifocal NMI bladder tumors recurring at a high frequency. After starting intravesical maintenance therapy with MMC the recurrence rate decreased and up to date the patient stayed recurrence free. Molecular analysis of the primary tumor revealed an S249C mutation in *FGFR3* (Figure 1B) and overexpression of FGFR3 (Figure 2B). LOH on chromosome 9 was detected by MA and confirmed by the genome wide SNP array analysis. We also found other regions of loss and gain that are considered minor when compared to tumors of the same stage and grade (Figure 3). There was no increased expression of TP53 (Figure 2C). Hence, this young patient appears to have the typical genetic changes found in older patients with NMI-BC. This implies that the patient could have a disease course comparable to older patients and warrants regular controls due to the risk of additional recurrences or progression. These findings combined with previous studies suggest that not only a patient's age, but also the molecular characteristics of the tumor determine the clinical disease course. Since it takes time to accumulate typical genetic changes involved in BC - e.g. mutations in *FGFR3* and *TP53* and LOH on chromosomes 8, 9, 10, 11, 17- leading to tumor formation, most BC patients will present at an older age. Possible explanations are that older patients have a longer exposure time to BC associated exogenous risk factors and secondly that pathophysiological changes in elderly causing urinary stasis in the bladder due to urine retention lead to an increased exposure to carcinogenic substances. We suggest that this could be the reason why tumors of young BC patients mostly have few genetic alterations and may represent a biologically distinct group of tumors with an overall good clinical disease course (Figure 4A). This is in concordance with one of the few molecular studies on BC in patients <19 years (n = 14) that found no mutations in *FGFR3*, no deletions on chromosome arms 9p, 9q or 17p, no MSI and only one mutation in *TP53* [7]. On the other hand other clinical studies of patients <40 years demonstrate a disease course comparable to older patients with typical aggressive behavior in the young presenting with a primary muscle invasive tumor, but unfortunately no molecular analyses of these tumors have been performed [2,5,12,13].

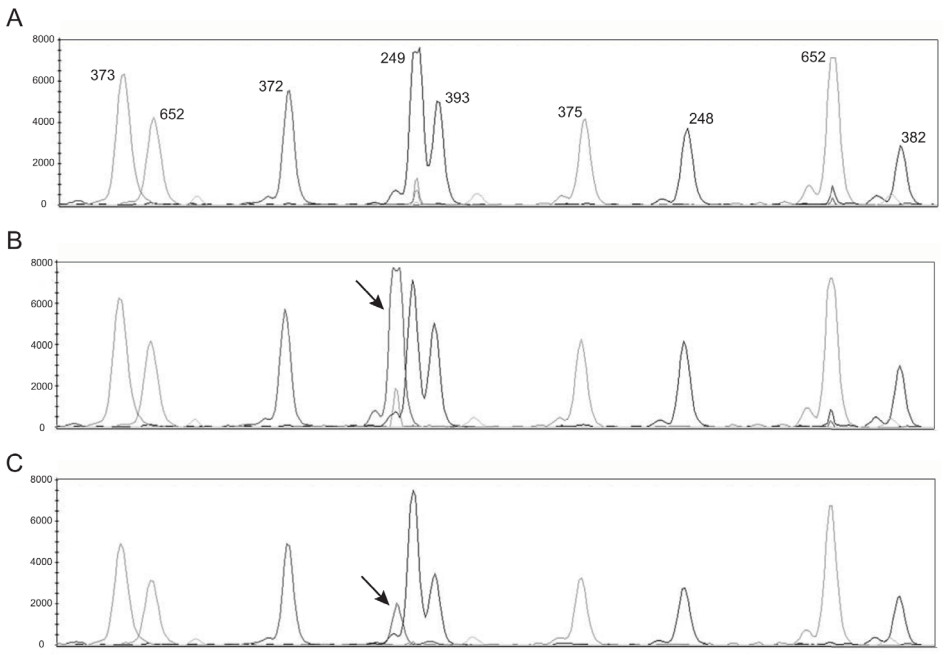

Figure 1 *FGFR3* mutation detection on urinary derived DNA. *FGFR3* mutation analysis of an *FGFR3* tumor without a mutation (A), mutation S249C on tumor DNA (B) and urinary derived DNA (C) from the same patient used in panel B.

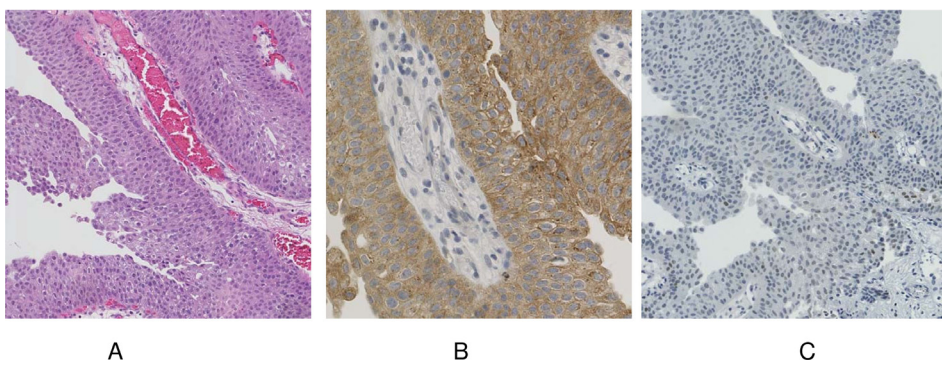

Figure 2 Protein expression levels of FGFR3 and TP53 in bladder tumor tissue. (A) Haematoxylin-Eosin staining of a papillary tumor. Original magnification ×20. (B) High levels of FGFR3 expression with a cytoplasmic and membranous patter. Original magnification ×40. (C) Low TP53 immunostaining of tumor cells showing a nuclear pattern. Original magnification ×20.

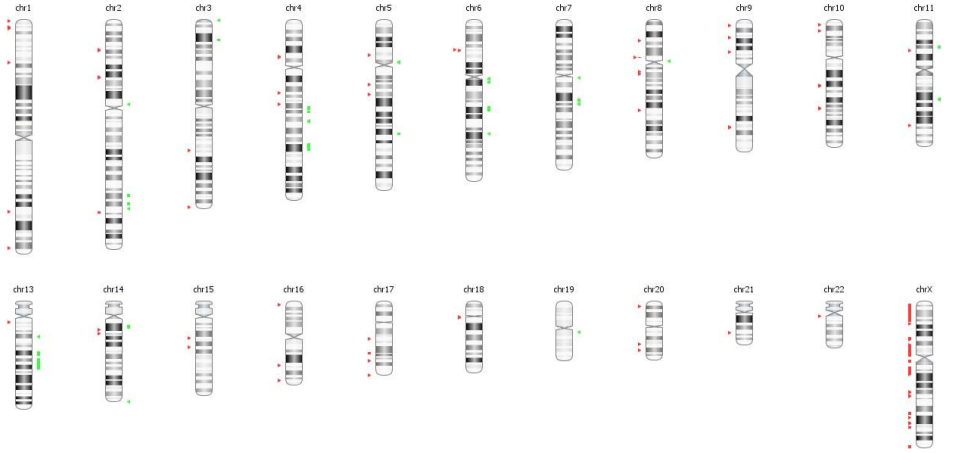

Figure 3 Overview of copy number alterations for all chromosomes. Red: loss, green: gain.

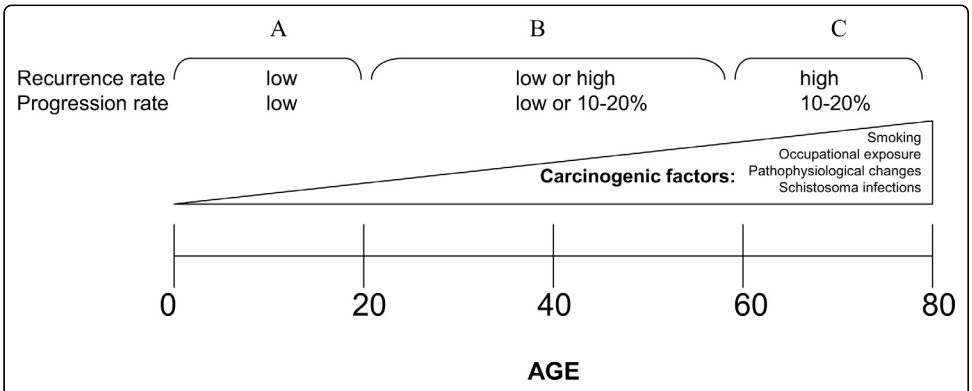

Figure 4 Age related occurrence of BC due to exposure to carcinogenic factors and BC-associated genetic changes. (A) Young BC patients, age <20, with a low carcinogenic exposure having chromosomal stable tumors, no mutations in *FGFR3* and *TP53* and no loss on chromosome 9. (B) Patients aged 20-60 with either a molecular profile corresponding to young (<20) or older (>60) patients. (C) Older BC patients with high exposure to carcinogenic factors and chromosomal unstable tumors, loss on chromosome 9, *FGFR3* and *TP53* mutations.

Exogenous risk factors that could have contributed to BC in our patient are smoking, exposure to diesel exhaust and depleted uranium (DU). First our patients smoking status is 5.5 pack-years, which is known to be associated with an increased risk of BC. Secondly, a meta-analysis of BC and diesel exhaust exposure demonstrated a relative risk of 1.44 for occupations exposed to high diesel fume levels [14]. Our patient worked as a driver of a diesel armed truck and was exposed to diesel exhaust fumes, working in a valley were the fumes were retained in a cloud of exploded ammunition. Lastly, although some believe that there is a link between exposure to DU and cancer development no hard evidence has been found to support this hypothesis. While evidence from Hiroshima data shows a latency period of 10-15 years to develop cancer this concerns an acute high-dose exposure and other studies were not able to demonstrate this link in Balkan veterans [15,16]. On the other hand two studies by Miller *et al*. demonstrated *in vitro* tumorigenic transformation of osteoblasts when exposed to DU [17,18].

Since Van der Aa *et al*. demonstrated that specifically young patients perceive a cystoscopic investigation as burdensome this emphasizes the need for patient stratification [19]. To determine whether a young BC patient should be monitored according to the standard follow-up protocol or can be monitored less frequently by cystoscopy, we propose to determine the *FGFR3* mutation status of the primary tumor. One possibility is that the tumor will have few genetic changes and secondly that the tumor will have genetic changes comparable to those found in older patients (Figure 4B, 4C). Mutations in *FGFR3* are tumor-specific and are not found in normal tissue, meaning that detection of a mutation in voided urine indicates the presence of tumor cells in the urinary tract. The results of the follow-up in time for our patient are indicated in Additional file 1. We demonstrate that the S249C mutation in *FGFR3* detected in the tumor was also detected in the urine (Figure 1C), indicating that the detected tumor cells were shed by the resected tumor. Our results demonstrate that urine cytology does not detect the tumor in two cases, which is in concordance with previous studies that demonstrate a low sensitivity of urine cytology for the detection of tumors of low stage and grade [20,21]. Although this concerns just one patient, our results imply that patient monitoring by *FGFR3* mutation analysis could be a feasible non-invasive method in the follow-up of young NMI-BC patients presenting with a mutation in *FGFR3* and indicate that future research is required to investigate this.

Conclusions

Multiple studies demonstrate the relatively benign disease course of bladder tumors in young patients, but there are some cases with a high recurrence rate and a progression rate comparable to older patients. We present a young patient having multiple multifocal recurrent bladder tumors with molecular characteristics found in older patients. It would be of interest to perform molecular studies in a larger subset of patients to elucidate whether these tumors comprise a biologically distinct group. Since BC tumors rarely present in young patients, a multicenter collaboration would be needed for investigation. This could ultimately lead to stratification of patients that need close monitoring and patients with favorable molecular characteristics of the tumor that can be monitored less frequently, hereby decreasing the number of cystoscopies performed in young patients who specifically perceive a cystoscopy as burdensome. For the follow-up of young patients presenting with an *FGFR3* mutant NMI tumor, *FGFR3* mutation analysis could be a feasible alternative for recurrence detection.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Additional file 1: Patient follow-up schedule in time. Patient's surveillance schedule starting at the primary tumor. Next rows represent tumor histology or urine cytology, *FGFR3* mutation status and type of intravesical treatment.
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[http://www.biomedcentral.com/content/supplementary/1471-2490-10-5-S1.DOC]

Acknowledgements
We would like to thank Dr. W. Kirkels for bringing this case to our attention and thank Dr. W. B. J. Nathrath for providing material of the primary tumor. This study was funded by a grant from the Koningin Wilhelmina Cancer foundation (KWF): 2006-3672.

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Authors' contributions
TCMZ and ECZ designed the study. TCMZ performed the literature review and drafted the manuscript. CSA, MB and TCMZ collected the tissue and urine material. CSA, SST and KvdK performed *FGFR3* mutation analysis and MA analysis. AvT performed the SNP-array and interpreted the results. MV performed immunohistochemical analysis and reviewed histopathological diagnosis. ECZ revised the manuscript for important intellectual content. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 10 September 2009 Accepted: 26 February 2010
Published: 26 February 2010

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CHAPTER 4

Optimization of non-muscle invasive bladder cancer recurrence detection using a urine-based FGFR3 mutation assay

J Urol. 2011 Aug; 186(2): 707-12

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Optimization of Nonmuscle Invasive Bladder Cancer Recurrence Detection Using a Urine Based *FGFR3* Mutation Assay

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Purpose: *FGFR3* mutations occur in 70% of nonmuscle invasive bladder tumors. Although urine based *FGFR3* mutation analysis can detect recurrence, its sensitivity may be limited if samples have few or no tumor cells. We determined whether test sensitivity depends on tumor size and the time point of urine collection, and how to increase sensitivity.

Materials and Methods: A total of 440 urine samples from 18 patients with a suspicious bladder lesion at cystoscopy were collected during 6 days before surgery. Eight patients (300 samples) had an *FGFR3* mutant tumor, including 4 each with a tumor greater than 3 and less than 1.5 cm. Polymerase chain reaction based *FGFR3* analysis was done on all tumors and urine samples.

Results: *FGFR3* mutations were detected in 257 of the 300 urine samples (86%) from patients with an *FGFR3* mutant tumor. Assay sensitivity was 100% for tumors greater than 3 cm and 75% for tumors less than 1.5 cm. It increased to 100% in patients with a less than 1.5 cm tumor when samples were pooled during 24 hours. Sensitivity was not influenced by the time of urine collection. All urine samples from patients with an *FGFR3* wild-type tumor were negative for *FGFR3* mutation.

Conclusions: The sensitivity of tumor detection increased with tumor size. *FGFR3* assay sensitivity depends on the number of shed tumor cells and improves by increasing urine volume. These findings suggest that there is an upper limit to the sensitivity of the *FGFR3* assay when 1 urine sample is analyzed. This may also apply to other DNA or RNA based assays.

Key Words: urinary bladder; urinary bladder neoplasms; tumor markers, biological; urine; mutation

Abbreviations and Acronyms

BC = bladder cancer

FGFR3 = fibroblast growth factor receptor-3

MT = *FGFR3* mutant

NMI = nonmuscle invasive

TUR = transurethral resection

TURB = bladder tumor TUR

WT = *FGFR3* wild-type

Approximately 80% of all patients with BC present with NMI tumors, of which 10% to 20% progress to muscle invasive disease. Since 70% of patients with NMI BC have at least 1 recurrence within 5 years, they are monitored by cystoscopy every 3 months for the first 2 years.[1] The high recurrence rate and stringent followup make BC from diagnosis to death the most costly cancer in the world.[2,3] In addition to high costs, the emotional distress of the patients is considerable.[4] To date the mentioned surveillance regimen has been the gold standard and its disadvantages emphasize the need to develop new diagnostic tools during followup.

Urine can be obtained noninvasively and used in inexpensive, simple assays. Many urinary biomarkers have been investigated, such as microsatellite instability using microsatellite analysis, nuclear matrix

protein-22, UroVysion® and the detection of mutations in *FGFR3* to detect recurrent BC.[5,6] Of patients with NMI BC 70% carry *FGFR3* mutations, which are associated with a good prognosis.[7–10] Thus, urine based *FGFR3* mutation analysis might serve as a biomarker for patients at low risk and to detect recurrent BC.[11–16] In most study protocols 1 urine sample is collected before TURB and the test outcome of this sample is used to determine assay sensitivity and specificity. However, since tumor cells are not always present in urine, the urine based assay is not 100% sensitive.

In accordance with this hypothesis our recent longitudinal study revealed *FGFR3* negative urine tests between a series of positive tests in multiple patients.[16] Therefore, we determined how often a urine test was *FGFR3* positive or negative in the days before a tumor was removed by TUR. We also determined whether assay sensitivity was related to tumor size. Our third objective was to determine the optimal frequency and time point of urine collection for *FGFR3* mutation analysis in voided urine. All urine specimens were collected during 6 days before surgery. Urine samples and concomitant tumors were analyzed for *FGFR3* mutations and the test results were compared to urine cytology results.

MATERIALS AND METHODS

Patients

A total of 18 patients with a suspect bladder lesion at cystoscopy who were scheduled for primary (6) or recurrent (12) TUR were included in our study at the outpatient departments of our 2 institutions. The Erasmus Medical Center medical ethical committee approved the study and written informed consent was obtained from all patients. The first 5 patients were selected to have a macroscopically visual tumor greater than 3 cm at cystoscopy and the second set was selected to have a tumor that was macroscopically less than 1.5 cm at cystoscopy. A prerequisite for study entry was patient mental capability to understand the study design and patient ability to collect all urine before surgery. Voided urine was collected during a minimum of 6 days before TURB. *FGFR3* mutation analysis and cytology were performed on the samples. Concomitant tumors were also analyzed for *FGFR3* mutations.

Urine Collection

Patients collected urine at each void and stored individual samples at 4C. Samples were collected twice daily a maximum of 24 hours before being processed individually. Urine samples were assessed for leukocytes, erythrocytes and nitrite using Multistix® 8 SG. Each sample was centrifuged for 10 minutes at 3,000 rpm (1,500 × gravity). Cell pellets were washed once with 10 ml phosphate buffered saline, resuspended in 1 ml phosphate buffered saline, transferred to an Eppendorf vial and centrifuged for 5 minutes at 6,000 rpm (3,000 × gravity). Supernatant was discarded and each cell pellet was stored at −20C until DNA isolation. DNA was extracted from the cell pellet using the QIAamp® DNA mini-kit according to the manufacturer protocol. *FGFR3* mutation analysis was performed on urinary DNA.

FGFR3 Mutation Analysis

Mutation assay was performed as described previously.[16,17] Briefly, we performed multiplex polymerase chain reaction of the 3 exons containing the most common *FGFR3* mutations (exons 7, 10 and 15). This was followed by single nucleotide polymorphism analysis using primers that anneal to the polymerase chain reaction product adjacent to the mutation site. Mutation probes were extended with a labeled dideoxynucleotide and products were analyzed on an ABI PRISM® 3100 Genetic Analyzer automatic sequencer with the label indicating the presence or absence of a mutation. GeneScan® Analysis Software, version 3.7 was used for data analysis.

Mutant and wild-type peak height is directly related to the relative presence of tumor cells and normal cells in voided urine. Hence, we used the ratio of these peaks to quantify the percent of mutant tumor cells in a urine sample by calculating the ratio of the height of the mutant to the corresponding wild-type peak.

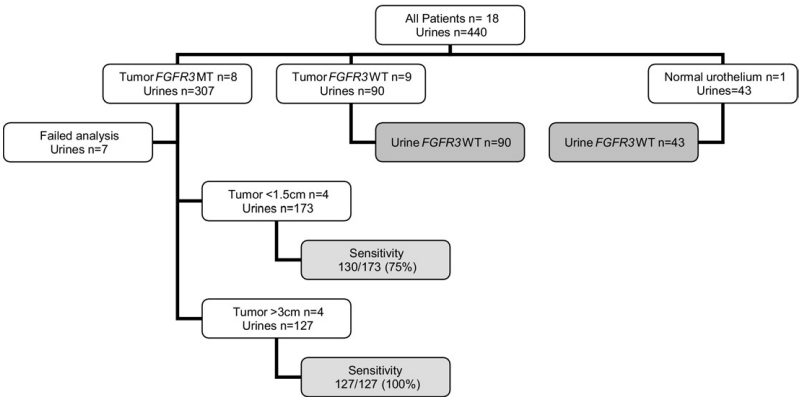
RESULTS

Sensitivity increased with tumor size and was not higher in morning urine.

The study included 18 patients scheduled for TUR. *FGFR3* mutation analysis was performed on resected material from the 18 patients and in a total of 440 associated urine samples. Eight patients had a MT tumor and all 307 urine samples from these patients were analyzed (see figure). *FGFR3* mutation analysis failed in 7 of the 307 urine samples (2%). For the other 9 patients with a WT tumor a total of 90 urine samples were analyzed. After TURB no tumor cells were detected in the resected tissue of patient 12 (43 urine samples) after TURB. All urine and tissue samples were analyzed by *FGFR3* mutation analysis. Table 1 shows the clinical and molecular characteristics of analyzed urine and tumor samples.

FGFR3 mutations were detected in 257 of 300 urine samples (86%) and undetected in 43 (14%). One *FGFR3* mutation was detected in dysplastic urothelium (table 2).

We then discriminated between tumors greater than 3 and less than 1.5 cm. MT cells were identified in all 127 urine samples (100%) from patients with a tumor greater than 3 cm and in 130 of 173 (75%) from patients with a tumor less than 1.5 cm (table 2). The average MT-to-WT peak height ratio in the urine of patients with tumors greater than 3 cm was higher than that of patients with tumors less than 1.5 cm (table 3). This might indicate that larger tumors shed more tumor cells into urine. Notably no tumors were detected by urine cytology (table 2).



Patients were divided by *FGFR3* status of study inclusion tumor and by tumor size less than 1.5 vs greater than 3 cm. *FGFR3* analysis sensitivity in patients with MT tumor attained 100% and 75% for tumors greater than 3 and less than 1.5 cm, respectively. No mutations were detected in urine of patients with WT tumor. One patient scheduled for tumor resection was tumor free after pathological examination of tissue.

The types of *FGFR3* mutations detected in patient urine were identical to those in the concomitant tumor, which strongly suggests that urinary MT tumor cells were shed by the resected tumor. Briefly, tumor cells were not always present in urine and the number of shed tumor cells increased with tumor size.

We then determined whether the sensitivity of our assay to detect tumors less than 1.5 cm could be increased by pooling urine samples collected within 24 hours. Table 4 shows that the average peak height of all urine specimens collected within 24 hours was always greater than zero, meaning that all tumors were detected.

Subsequently to determine whether morning urine contained more tumor cells due to the overnight concentration of urinary cells in the bladder we compared the MT-to-WT peak ratio of the first urine sample in the morning to the ratio of all other urine samples (morning urine). Table 3 shows that the average peak ratio of the first morning urine was generally lower than the ratio of the other urine samples. Based on these data we conclude that the first morning urine does not necessarily contain more tumor cells than urine collected during the day.

In 1 of the 18 study patients no tumor cells were detected in the tissue removed at TURB. Cystoscopy

Table 1. *Clinical and molecular characteristics of 18 patients*

Pt No.—Sex—Age at Diagnosis	Tumor Type	Multiplicity	Size (cm)	Stage/Grade	Tumor *FGFR3*
1— M—73	Primary	Multiple	Greater than 3	Ta/G2	S249C + Y375C
2— M—66	Primary	Multiple	Greater than 3	Ta/G2	S249C
4— F—84	Primary	Multiple	Greater than 3	Ta/G1	Y375C
5— F—58	Recurrent	Solitary	Greater than 3	Ta/G2	S249C
9— M—26	Recurrent	Multiple	Less than 1.5	Ta/G2	S249C
13— M—74	Recurrent	Solitary	Less than 1.5	Ta/G2	S249C
16— M—66	Recurrent	Solitary	Less than 1.5	Ta/G2	S249C
17— M—61	Recurrent	Solitary	Less than 1.5	Dysplasia	R248C
3— M—72	Recurrent	Multiple	Greater than 3	Ta/G2	WT
6— M—79	Recurrent	Multiple	Less than 1.5	Ta/G2	WT
7— M—64	Recurrent	Multiple	Less than 1.5	Ta/G2	WT
8— M—85	Recurrent	Multiple	Less than 1.5	Ta/G2	WT
10— M—57	Primary	Solitary	Less than 1.5	Ta/G1	WT
11— M—67	Recurrent	Multiple	Less than 1.5	Ta/G1	WT
12— M—77	Recurrent	None	Less than 1.5	—	WT
14— M—84	Recurrent	Multiple	Less than 1.5	Ta/G2	WT
15— M—66	Primary	Solitary	Less than 1.5	Ta/G1	WT
18— M—56	Primary	Solitary	Less than 1.5	Ta/G2	WT

Table 2. *Sensitivity to detect FGFR3 mutation in urine when cytology showed no malignancy*

Pt No.	Tumor *FGFR3*	Urine Samples *FGFR3*	No. Analyzed	No. *FGFR3* Pos (% sensitivity)
Tumor greater than 3 cm:				
1	S249C + Y375C	S249C + Y375C	16	16 (100)
2	S249C	S249C	31	31 (100)
4	Y375C	Y375C	39	39 (100)
5	S249C	S249C	41	41 (100)
Tumor less than 1.5 cm:				
9	S249C	S249C	24	16 (67)
13	S249C	S249C	30	26 (87)
16	S249C	S249C	68	56 (82)
17	R248C	R248C	51	32 (63)

revealed a suspect elevated lesion on the anterior bladder wall. Accordingly all 43 urine samples were negative for MT (see figure). The primary tumor of this patient had previously been found to have an S249C mutation in the *FGFR3* gene. As a control, we also analyzed 10 urine samples from each patient with a WT tumor for a total of 90 samples (see figure). No mutations were detected in these urine samples. These findings confirm the high specificity of the *FGFR3* urine assay.[16]

DISCUSSION

FGFR3 mutations are found in 70% of all patients presenting with an NMI bladder tumor. These mutations are tumor specific and absent in the urine of patients without BC or normal urothelium.[16,18] These findings make *FGFR3* mutation analysis on voided urine an optimal urine based assay for followup.

Previously we noted that *FGFR3* mutation detection on voided urine might be feasible during followup. A repeat finding was *FGFR3* negative urine tests between a series of positive test results, which could imply the scarcity or absence of tumor cells in some samples.[16] Thus, we determined the prevalence of *FGFR3* positive and negative urine samples preceding the removal of a MT tumor. We also investigated whether tumor size could influence assay sensitivity and determined the optimal frequency and time point of urine collection. *FGFR3* mutations in the urine of patients with a tumor greater than 3 cm were detected in all samples for 100% sensitivity compared to 75% sensitivity (range 63% to 87%) for tumors less than 1.5 cm in patients with a MT tumor. Thus, we suggest that the sensitivity of detection depends on tumor size.

Table 3. *MT-to-WT ratio of peak height of morning urine sample vs other samples*

Pt No.	No. Urine Samples (av ratio) Overall	Morning	Day/Night
Tumor greater than 3 cm:			
1	16 (0.17)	4 (0.16)	12 (0.18)
2	31 (0.79)	6 (0.45)	25 (0.87)
4	39 (0.89)	6 (0.78)	33 (0.91)
5	41 (0.99)	8 (0.95)	33 (1.00)
Tumor less than 1.5 cm:			
9	24 (0.15)	5 (0.24)	19 (0.13)
13	30 (0.17)	5 (0.06)	25 (0.19)
16	68 (0.20)	5 (0.18)	64 (0.20)
17	51 (0.09)	8 (0.04)	43 (0.09)

Table 4. *Mutant peak height in pooled 24-hour urine samples*

Day	Av 24-Hr Peak Ht Pt 1	Pt 2	Pt 4	Pt 5	Pt 9	Pt 13	Pt 16	Pt 17
1	324	2,609	4,557	8,139	774	403	702	24
2	367	1,599	3,880	4,439	63	467	1,052	13
3	123	917	4,082	7,170	519	263	2,027	31
4	546	1,023	2,642	7,492	1,091	354	917	121
5	1,090	1,631	2,452	7,417	91	295	1,453	94
6		2,588	4,399	8,014				115
7		826	2,834	5,131				72
8				5,994				92
9				8,388				

Lower sensitivity in tumors less than 1.5 cm could have been caused by a low number or the absence of tumor cells in the urine. Extrapolating the results to all urine based assays that depend on the number of urinary tumor cells, this suggests that the sensitivity of the most potent urine based assays, such as MT analysis (81%), microsatellite analysis (58%), fluorescence in situ hybridization (76%) and ImmunoCyt™ (84%), will never attain 100%.[16,19,20] A possible explanation for a lower number or the absence of tumor cells in the urine of patients with tumors less than 1.5 cm could be that they shed fewer cells due to less mechanical stress on the tumor. Our group also noted this in a recent study in which sensitivity to detect primary tumors, which on average are large, was higher than sensitivity to detect recurrent bladder tumors in voided urine (unpublished data). Also, a possibility could be that smaller tumors shed fewer cells due to different biological characteristics of the tumor. Lastly, the detection threshold of the urine based assay might be insufficient when there are few tumor cells. However, as reported previously, urinary *FGFR3* mutations can be detected in the presence of 95% normal cells and, thus, it seems unlikely that the assay detection threshold could completely explain these findings.[16]

In 1 patient the pathological classification of the tumor was dysplasia. Pathology reports were reviewed and confirmed by an expert uropathologist. Since to date *FGFR3* mutations have only been

detected in urothelial cell carcinoma and flat urothelial hyperplasia,[21] to our knowledge this is the first study of an *FGFR3* mutation in dysplastic urothelial tissue.

We then determined that morning urine, which is assumed to be concentrated overnight and, therefore, is expected to contain more tumor cells, did not contain the highest number of neoplastic cells (table 3). A possible explanation is that most of these elderly patients had polyuria or nocturia caused by comorbidity, such as benign prostate hyperplasia or diabetes mellitus. Hence, in these patients there is simply not enough time to collect more tumor cells in urine. In line with this finding the youngest patient (patient 9), who was 26 years old and did not have nocturia or polyuria, had higher average peak height ratios in the first morning urine than in urine collected during the day. Larger cohorts of young patients are needed to confirm this finding, especially since young patients perceive cystoscopy to be burdensome and would specifically benefit from a decreased number of cystoscopies.[4]

Since 24-hour urine collection is already a standard clinical procedure, we observed that this potentially improved mutation assay sensitivity from 75% to 100% in patients with tumors less than 1.5 cm (table 4). At the time of this study Urine Preservation Solution (Copan Italia, Brescia, Italy) was being tested. Results have shown that urinary cells and DNA are stable at room temperature for 2 weeks (data not shown), eliminating the need for sample cooling and direct processing.

Urine cytology was also done and no tumors were detected by cytology. This finding is in accordance with previous studies showing the low sensitivity of cytology for low stage, low grade tumors.[20,22–24]

Our study included 1 patient who had positive cystoscopy but whose tissue removed at TURB was free of any tumor cells. It was known from a previous study that this patient had a MT primary tumor but no mutations were detected in any analyzed urine sample. Also, a total of 90 urine samples from 9 patients with a WT tumor were also negative for *FGFR3* mutation. Together these findings underline the high specificity of the assay. Based on these and previous results we suggest that patients can be stratified by the *FGFR3* mutation status of the primary tumor. Patients with a MT tumor should undergo cystoscopy at 3, 12 and 24 months, and urinary *FGFR3* mutation analysis at 6, 9, 15, 18, 21 and 27 months for the first 2 years.[16] Urine should be collected during 24 hours to achieve the highest sensitivity of *FGFR3* mutation analysis.

We suggest that a positive test should be followed by cystoscopy since in most positive test results recurrence is found, especially when the urologist is aware of the positive test outcome.[16,25] When the urine test is negative in patients with a MT primary tumor, no cystoscopy is indicated if the urine is tested again after 3 and 6 months. We previously noted that 19% of primary MT tumors are followed by WT recurrence. Also, we observed that wild-type recurrence in patients with a mutant primary tumor had lower stage and grade than recurrence in patients presenting with a wild-type tumor.

Hence, we have no indication that wild-type recurrence following a mutant primary tumor is associated with disease progression. Thus, we suggest that it would be safe to repeat urine testing after a negative test, especially since recurrence develops during an extended period and multiple urine tests can be done during this period, as reported by Kompier et al.[10] Obviously after a grade 3 recurrence is found frequent cystoscopy is warranted. We suggest other methods of followup for patients with a primary WT tumor, for example detection of loss of heterozygosity.[6]

Strengths of the study include *FGFR3* mutation analysis of a large number of urine samples together with the concomitant recurrent tumor in patients with a MT tumor as well as control patients with a WT tumor. The study could have been improved by validating the results in an independent, larger patient cohort and by collecting samples for 24 hours instead of pooling urine. Also, random inclusion of patients without prior selection criteria at cystoscopy might have strengthened our findings.

Briefly, followup by urinary *FGFR3* mutation analysis might improve patient quality of life, decrease the number of cystoscopies and lower treatment associated costs. We report that tumor cells are not always present in the urine of patients with tumors less than 1.5 cm, which negatively affects assay sensitivity. This problem could be solved by 24-hour urine collection. It would be interesting to perform a larger trial with urine samples pooled during a 24-hour period to validate these findings.

CONCLUSIONS

Sensitivity of the urine based *FGFR3* mutation detection assay increases with tumor size and is not influenced by the time that urine was collected. We confirm that urine may contain few or no tumor cells, affecting assay sensitivity. Increasing the volume of collected urine could solve this. Our findings suggest that there is an upper limit to sensitivity due to few or no tumor cells in urine, which may also apply to other DNA or RNA based urine assays.

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CHAPTER 5

No evidence of FGFR3 mutations in prostate cancer

Prostate. 2011 May;71(6):637-41

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No Evidence of *FGFR3* Mutations in Prostate Cancer

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BACKGROUND. *FGFR3* mutations are associated with a good clinical disease course in bladder tumors. Currently, prognostic markers to stratify prostate cancer (PCa) patients for conservative management are lacking. Conflicting results have been found on the presence of *FGFR3* mutations in PCa. Our objective was to determine the prevalence of *FGFR3* mutations in a subset of prostate tumors. Next, determine the prevalence of *FGFR3* mutations in PCa patients with coexistent tumors in other tissues.

METHODS. Primary and locally advanced prostate tumors (n = 132) were collected at our medical center. From the 132 PCa patients, 28 (21%) were diagnosed with coexistent primary tumors (bladder, skin, pancreas, renal cell, gastric, colon, hepatic, and lung). Tumors were analyzed by *FGFR3* mutation analysis on exon 7, 10, and 15, known to harbor the most frequent mutations.

RESULTS. The prevalence of *FGFR3* mutations in patients with only PCa was 0%. Most PCa patients presented with coexistent bladder (n = 12) and bladder and skin tumors (n = 7). Other coexistent tumors in PCa patients included: bladder and pancreatic cancer (n = 1); bladder and renal cell carcinoma (n = 1); bladder and gastric carcinoma (n = 1); skin cancer (n = 1); colon cancer (n = 3); hepatic carcinoma (n = 1); and lung cancer (n = 1). *FGFR3* mutations were detected in 9/15 (60%) analyzed bladder tumors.

CONCLUSIONS. *FGFR3* mutations were absent in the investigated prostate tumors, suggesting a minor role of these mutations in tumorigenesis. Hence, *FGFR3* mutation analysis is not suitable to select patients for conservative management. Interestingly, if a prostate tumor coincided with other tumors these were mostly bladder and skin. *Prostate 71: 637–641, 2011.*

KEY WORDS: ***FGFR3*; mutation; prostate cancer**

INTRODUCTION

Prostate cancer (PCa) is the second most common cause of cancer-related death in men [1]. Interestingly, PCa is present in 80% of men over the age of 80 but it will only lead to disease-related symptoms in a minority of the patients.

Currently, prognostic markers to stratify patients at a high risk of disease progression are scarce. Hence, there is a need for development of new biomarkers. Previous investigations demonstrated the role of the FGF-axis in PCa carcinogenesis. Decreased expression of fibroblast growth factor receptor-2 (*FGFR2*) mRNA was found in androgen-independent tumors and was associated with progression [2]. Overexpression of *FGFR4* mRNA and protein was shown to be associated with high-grade tumors and a poor disease-specific survival [3–5]. The role of FGFR3 in PCa remains largely unknown. Two independent studies demonstrated similar levels of expression of *FGFR3* in benign prostatic hyperplasia (BPH) and PCa samples [3,5]. Mutations in *FGFR3* have been found in multiple myeloma, benign skin tumors, colon, cervical, oral and

The authors have no conflict of interest to disclose.

Grant sponsor: Koningin Wilhelmina Fonds (KWF); Grant number: 2006-3672.

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Received 26 July 2010; Accepted 27 August 2010
DOI 10.1002/pros.21279
Published online 18 October 2010 in Wiley Online Library (wileyonlinelibrary.com).

bladder cancer [6–10]. *FGFR3* mutations in bladder tumors were shown to be associated with a good clinical prognosis and an *FGFR3*-based stratification of patients demonstrated to be feasible during follow-up [11–14]. Two small studies reported on the absence of *FGFR3* mutations in PCa. In contrast, Hernandez et al. [15] recently demonstrated a low prevalence (9%) of *FGFR3* mutations in a large group of prostate tumors and the association of *FGFR3* mutations with low-grade PCa, suggesting a tool for identification of patients with a good prognosis. As this finding could contribute to an improved selection of patients suitable for conservative management, we aimed to determine the prevalence of *FGFR3* mutations in a large set of prostate tumors. In the abovementioned study by Hernandez et al., it was demonstrated that *FGFR3* mutations were mostly present in PCa patients with coexistent bladder or skin tumors. Hence, we focused on the prevalence of *FGFR3* mutations in PCa patients with coexistent tumors in other tissues.

MATERIALS AND METHODS

Patients and Sample Collection

Genomic DNA was available from 132 clinical PCa samples. Eighty-one samples were primary prostate tumors obtained by radical prostatectomy, 1 sample was obtained by cystoprostatectomy, 17 samples were needle biopsies, and 33 samples were locally advanced or recurrent tumors obtained by transurethral resection of the prostate (TURP) at Erasmus MC Rotterdam, the Netherlands. In the latter, TURP was done for reasons of bladder outlet obstruction due to local progression. The vast majority of the recurrent tumor samples were taken during endocrine therapy. Not surprisingly, well-differentiated tumors were absent in the TURP group. From the 132 PCa patients 28 (21%) were known with coexistent primary tumors (bladder, skin, pancreas, renal cell, gastric, colon, hepatic, and lung).

DNA Extraction

Hematoxilin/eosin-stained tissue sections from prostate tumors were histologically evaluated by two pathologists (van der Kwast, van Leenders. Only samples that contained at least 70% tumor cells were selected for analysis. Tissues were snap-frozen and stored in liquid nitrogen. Genomic DNA was isolated using the Puregen system from Gentra Systems (Minneapolis, MN) according to the manufacturer's instructions. Use of the samples for research purposes was approved by the Erasmus MC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

FGFR3 Mutation Analysis

A multiplex PCR of the three regions that contain the most frequent *FGFR3* mutations was performed as described previously [14,16]. These regions comprise the codon mutations: R248C and S249C (exon 7), G372C, S373C, Y375C, G382R, and A393E (exon 10), and K652M, K652T, K652E, and K652Q (exon 15). PCR was performed using 15 μl containing 1–5 ng genomic DNA, 1× PCR buffer (Promega, Madison, WI), 1.5 mmol/L $MgCl_2$, 0.17 mmol/L dNTPs (Roche, Basel, Switzerland), 1.0 U Taq polymerase (Promega), 5% glycerol (Fluka, Buchs SG, Switzerland), 18 pmol of exon 7 primers, 7.5 pmol of exon 10 primers, and 10 pmol of exon 15 primers. Cycling conditions were: 5 min at 95°C, 35 cycles at 95°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec, followed by 10 min at 72°C. The PCR product was treated with 3 U of shrimp alkaline phosphatase (Amersham Biosciences, Uppsala, Sweden) and 2 U of Exonuclease I (Amersham Biosciences) to remove excess primers and dNTPs. This was followed by a mutation analysis (ABI PRISM SNaPshot Multiplex Kit, Applied Biosystems, Foster City, CA) using probes that anneal to the PCR product adjacent to the mutation site. Different lengths of poly (dT) tails were attached to the 5′-end to enable simultaneous detection on the sequencer. The mutation detection reaction was performed in 10 μl, containing 1 μl of multiplex PCR product, 2.5 μl Ready reaction mix, 1× sequencing buffer and probes as shown in Supplementary Material S2. Cycling conditions were 35 cycles of rapid thermal ramp (1.30) to 96°C, 96°C for 10 sec, rapid thermal ramp (1.30) to 58.5°C, and 58.5 for 40 sec, followed by treatment with 1 U shrimp alkaline phosphatase. Separation was in a 30-min run on 36-cm long capillaries on an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems) with the label indicating the presence or absence of a mutation.

RESULTS

We analyzed 132 prostate tumors for the presence of mutations in *FGFR3*, but no mutations were detected in any of the samples. Clinicopathological data were available for the cohort of primary prostate tumors (n = 81). The mean age at diagnosis was 61.9 years (SD ± 5.4) and the mean PSA at diagnosis was 15.7 ng/ml (SD ± 21.5). Clinicopathological characteristics of the primary tumors are listed in Table I.

Next, we investigated the prevalence of *FGFR3* mutations in a subgroup of PCa patients (28/132) with other coexistent tumors (Table II). Most PCa patients presented with coexistent bladder (n = 12) and bladder and skin (n = 7) tumors. Other coexistent tumors included: bladder and pancreatic cancer (n = 1);

TABLE I. Clinicopathological Characteristics of the Primary Tumors

Characteristic		
Mean age (±SD)	61.9	±5.4 years
Mean PSA (±SD)	15.7	±21.5 ng/ml
cT-stage		
Organ confined tumor	55	61.7%
Extraprostatic tumor	16	19.8%
Unknown	10	13.3%
pT-stage		
≤pT2c	25	30.9%
≥pT3a	53	65.4%
Unknown	3	3.7%
Surgical margins		
Positive	38	46.9%
Negative	39	48.1%
Unknown	4	4.9%
Gleason score		
<7	46	56.8%
=7	21	25.9%
>7	14	17.3%
Occult metastases at RP		
Yes	10	12.3%
No	66	81.5%
Unknown	5	6.2%

bladder and renal cell carcinoma (n = 1); bladder and gastric carcinoma (n = 1); skin cancer (n = 1); colon cancer (n = 3); hepatic carcinoma (n = 1); and lung cancer (n = 1). *FGFR3* mutations were detected in 9/15 (60%) analyzed bladder tumors (Table III). All *FGFR3* mutant bladder tumors contained the S249C mutation and one patient had an S249C and Y375C mutation. No *FGFR3* mutations were detected in the skin, pancreatic, renal cell, gastric, colon, hepatic, and lung tumors.

DISCUSSION

Due to PSA-based screening there has been an increase in the detection of PCa over the past two decades. It is estimated that PCa affects 80% of all men over 80 years of age but only a minority of these patients will have clinical symptoms during their lifetime. Recently, it was shown that screening for PCa reduces cancer-specific mortality but at the cost of overdiagnosis and overtreatment [17]. Currently, there are no specific biomarkers available that can identify patients at a low risk of progression. Such a marker could aid in the selection of patients who are suitable for conservative management and thereby reducing the negative sides of treatment and improving patient quality of life. Mutations in *FGFR3* have mainly been found in low-grade bladder tumors (70%) and benign skin (25–85%) tumors [12,18–20]. Specifically in bladder tumors, *FGFR3* mutations have been associated with a good prognosis. Our objective was to determine the prevalence of *FGFR3* mutations in a large set of PCa tumors.

FGFR3 mutations were not detected in any of the analyzed prostate tumors (n = 132). In contrast to our findings, Hernandez et al. recently reported *FGFR3* mutations in 9/112 (8%) prostate tumors. Stratifying the cases according to the Gleason score showed an association of *FGFR3* mutations with low-grade prostate tumors (GS = 6; 8/9, 89%). Thus, they concluded that mutations in *FGFR3* were associated with a subset of prostate tumors with a low Gleason score and a good prognosis. Furthermore, Naimi et al. [21] and Sibley et al. [22] investigated the prevalence of *FGFR3* mutations in prostate tumors but found no mutations. It is possible that these patient cohorts (n = 20 and n = 10, respectively) were too small to detect *FGFR3* mutations in the prostate tumors, which seems not the case for our cohort (n = 132). There are multiple factors that could explain the observed discrepancies. First, our study included 17 needle biopsies and it is well established that needle biopsies contain relatively low number of tumor cells, meaning that our results could include false negatives. However, the assay used can detect *FGFR3* mutations if only 5% tumor cells are present, so it seems unlikely that the sensitivity of the

TABLE II. Patient Numbers According to the Different Tumor Types

n = 132	Type of tumors	n
Group 1 (n = 104)	Prostate	104
Group 2 (n = 28)	Prostate + bladder	12
	Prostate + bladder + skin	7
	Prostate + bladder + pancreas	1
	Prostate + bladder + renal cell carcinoma	1
	Prostate + bladder + gastric carcinoma	1
	Prostate + skin	1
	Prostate + colon	3
	Prostate + hepatocelluar carcinoma	1
	Prostate + lung	1

TABLE III. Outcome *FGFR3* mutation analysis in patients with PCa and coexistent tumors

Sample ID	Sample type	Other tumors	FGFR3 Pca	FGFR3 BC	FGFR3 other
G-204	Prostatectomy	Bladder, TaG1	WT	N/A	
24	Biopsy	Bladder, TaG2	WT	S249C	—
111	Biopsy	Bladder, TaG2	WT	S249C	
119	Biopsy	Bladder, T1G3 +CIS	WT	WT	—
326	Biopsy	Bladder, TaG2	WT	N/A	—
706	Biopsy	Bladder, TaG2 + CIS	N/A	WT	
729	Biopsy	Bladder, TaG3 + CIS	WT	WT	—
743	Cystoprostatectomy	Bladder, T3G3 +CIS	WT	N/A	—
761	Biopsy	Bladder, T2G3	WT	N/A	
890	Biopsy	Bladder, TaG2	N/A	S249C/Y375C	—
891	Biopsy	Bladder, TaG2	N/A	S249C	—
T4-5	TURT	Bladder, TaG1	WT	N/A	
G-289	Prostatectomy	Bladder TaG2, basal cell carcinoma	WT	S249C	WT
115	Biopsy	Bladder TaG1, basal cell carcinoma	WT	Y375C	WT
131	Biopsy	Bladder TaG2, basal cell carcinoma	WT	S249C	WT
766	Biopsy	Bladder TaG1, basal cell carcinoma	WT	WT	WT
46	Biopsy	Bladder T1G3, basal cell carcinoma	N/A	S249C	N/A
371	TURT	Bladder TaG2, seborrheic keratosis	N/A	N/A	N/A
79	Biopsy	Bladder T1G3, hand papilloma	WT	WT	N/A
105	Biopsy	Bladder T1G2, pancreatic carcinoma	WT	WT	N/A
721	Biopsy	Bladder TaG2, renal cell carcinoma	WT	N/A	N/A
677	Biopsy	Bladder T1G3, gastric cancer	N/A	S249C	WT
G-051	Prostatectomy	Basal cell carcinoma, actinic keratosis, squamous cell carcinoma skin	WT	N/A	N/A
G-165	Prostatectomy	Colon cancer	WT		N/A
G-272	Prostatectomy	Colon cancer	WT	—	N/A
G-255	Prostatectomy	Colon cancer	WT		N/A
G-124	Prostatectomy	Hepatic cancer	WT		N/A
PC300	TURT	Lung cancer	WT	—	N/A

PCa, prostate cancer; BC, Bladder cancer; TURT, trans-urethral resection of the tumor; CIS, carcinoma in situ; WT, FGFR3 wild-type; N/A, not available.

assay could explain the absence of mutations [14]. The study by Hernandez et al. included 25/112 needle biopsies without *FGFR3* mutations. Secondly, contamination of morphologically normal, but molecular aberrant urothelial cells from the prostatic urethra and prostatic ducts could be a cause of false positives in the study by Hernandez et al. Therefore, it would be of interest to determine whether the *FGFR3*-positive tumors are located in the transitional zone of the prostate. Lastly, it cannot be excluded that Spanish patients differ from those in Northern Europe.

Next, we determined the prevalence of *FGFR3* mutations in PCa patients with coexistent tumors. *FGFR3* mutations were only detected in bladder tumors. Hernandez et al. did not find any *FGFR3* mutations in their bladder tumors, which could be explained by the inclusion of mainly high-grade muscle-invasive tumors that have a low prevalence of *FGFR3* mutations. It is an interesting finding that prostate tumors mostly coincide with bladder and skin tumors although it should be mentioned that this is a selected group and it would be interesting to confirm this finding in a large unselected group of patients. One patient had coexistent prostate, bladder and lung cancer. Smoking is an important risk factor for lung and bladder cancer and could represent a common cause of these tumor types. In addition to *FGFR3* mutations, other genes like Patched Homolog (*PTCH*), Tuberous Sclerosis-1 (*TSC1*), and members from the PI3K/PTEN/AKT and Ras/Raf/MAPK pathway have been indicated in the tumorigenesis of urothelial, skin and prostate tumors and it would be of interest to determine the mutation status of these genes in coexistent tumors [23,24].

In conclusion, *FGFR3* mutations were not detected in PCa tumors. Interestingly, we confirmed the presence of mostly coexistent prostate, bladder and skin tumors in this selected group. This is an interesting finding since *FGFR3* mutations are found in low-grade bladder tumors, benign epidermal nevi and seborrheic keratosis [19,20,23]. Larger studies on the prevalence of coexistent tumors in one patient and investigation of

molecular alterations in different pathways are needed to prove a possible relationship of tumor development.

CONCLUSIONS

Mutations in *FGFR3* were not found in PCa, hence stratification of patients for conservative management by *FGFR3* analysis is not suitable. Interestingly, if a prostate tumor coincides with other tumors these are mostly bladder and skin.

ACKNOWLEDGMENTS

This study was funded by a grant from the Koningin Wilhelmina Cancer foundation (KWF): 2006-3672.

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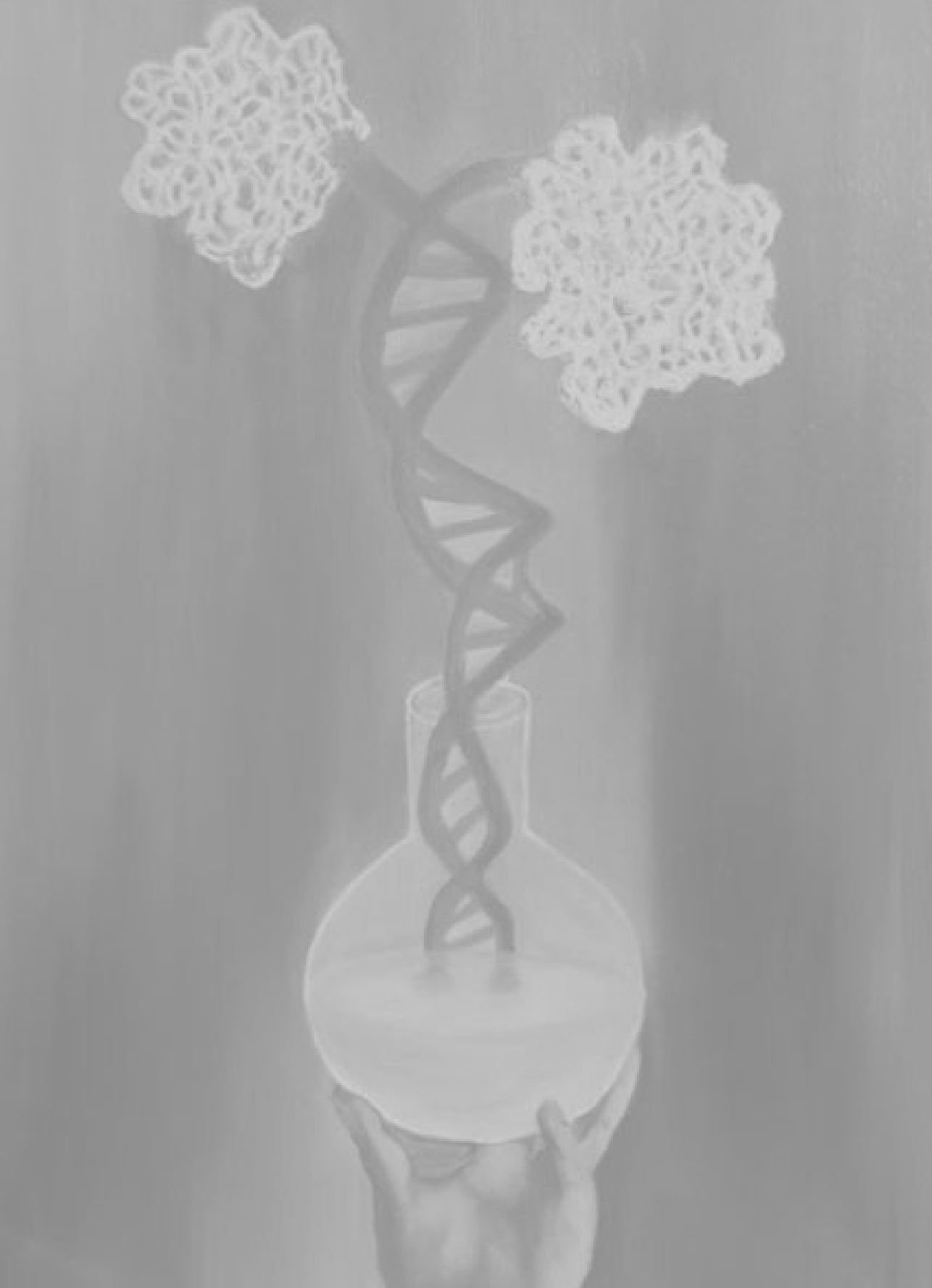

PART III

SURVEILLANCE OF PATIENTS WITH LOW-GRADE NON-MUSCLE-INVASIVE BLADDER CANCER

Based on:

Chapter 6

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

Chapter 7

Combinations of Urinary Biomarkers for Surveillance of Patients with Incident Nonmuscle Invasive Bladder Cancer: The European FP7 UROMOL Project

CHAPTER 6

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

BJU Int. 2012 Mar;109(6):941-8

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

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Accepted for publication 16 March 2011

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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.

PATIENTS AND METHODS

- Genes frequently methylated in NMIBC tumours ($n = 37$) were selected to develop a BC-specific MS-MLPA assay.
- Genes methylated in blood from patientswith BC ($n = 29$) and genes methylated in urine from patients with no history of BC ($n = 46$) 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 from patients with a histologically confirmed recurrence ($n = 68$ test set; $n = 49$ validation set) and urine samples from patients without BC ($n = 91$, test set) and urine from recurrence-free BC (rec-free BC) patients ($n = 60$, validation set).
- A model was developed to predict the probability of having a recurrence based on methylation of the four-gene panel and a threshold probability with the highest sensitivity and specificity was determined.
- The 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_a*, *TERT_a*, *TERT_b*, and *EDNRB*. This panel reached an area under the receiver operating characteristic curve (AUC) of 0.82 (test set) and AUC 0.69 (validation set). Sensitivity and specificity for the detection of a concomitant tumour 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 in patients with NMIBC in voided urine.
- The findings are promising and improvement of this test could eventually contribute to a more individualized patient friendly surveillance.

What's known on the subject? and What does the study add?
Multiple studies report on the detection of methylation in voided urine samples as a possible approach for the follow-up of non-muscle invasive bladder cancer patients. Previous studies analyze methylation gene panels in a mixture of primary and recurrent tumours. As primary tumours are larger than recurrent tumours and thus easier to detect in urine, validation of methylation markers in urine samples from patients with primary tumours will result in a test sensitivity that does not reflect the true sensitivity of the assay.

This study is the first to select a subset of genes specifically methylated in non-muscle invasive bladder cancer recurrences and validates the gene panel in two independent sets of urine samples from recurrent patients, thus simulating the disease course according to the clinical presentation.

KEYWORDS

bladder cancer, urine, methylation, surveillance

INTRODUCTION

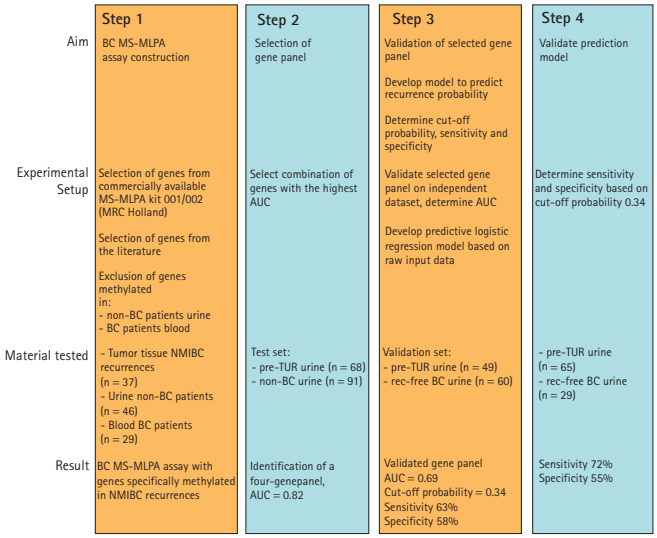
Bladder cancer (BC) is the most prevalent type of urothelial cell carcinoma and is associated with the highest costs in patient surveillance compared withother types of cancer [1]. This is mainly caused by the high recurrence rate of non-muscle invasive BC (NMIBC), which necessitates life-long cystoscopic follow-up and frequent transurethral resections (TURs) [2–4]. Today, cystoscopy remains the 'gold standard' for surveillance of patients with NMIBC, but this is a costly and time-consuming procedure with a sensitivity of 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 tumours, 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, consequently 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 the identification of new urinary biomarkers for the follow-up of patients with NMIBC.

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 have shown the role of aberrant DNA promoter hypermethylation, leading to transcriptional silencing of tumour suppressor genes [15–17]. Hypermethylation in BC has been associated with age, smoking status, gender, tumour location, stage, recurrence rate and progression [18–22]. In addition to detection of methylation in tumour tissue, aberrant methylation has also been detected in voided urine of patients with BC and appears to be more sensitive than urine cytology [23–30].

Current urinary biomarkers are mostly tested and validated on primary tumours, which are large in size and thus easier to detect in urine than in recurrent tumours. In addition to this, muscle-invasive (MI) tumours are used to validate markers for the detection of recurrent bladder tumours. MI tumours are also larger in size, are high grade with more genetic aberrations and shed more tumour cells than NMI tumours. 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 patients with NMIBC. 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 tumour cells. Lastly, we determined the sensitivity and specificity of the selected gene panel on an independent test and validation cohort.

FIG. 1. Four-step process to develop the BC MS-MLPA assay. 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 beforetransurethral resection of tumour.



PATIENTS AND METHODS

The study design consisted of a four-step process to develop the MS-MLPA specifically for the detection of NMIBC recurrent tumours (Fig. 1, Table 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.

Step 1. Recurrent tumours from patients included with a primary NMI bladder tumour 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 before cystoscopy at the Erasmus MC Urology Outpatient Department and stored at 4 °C. All patients with a history of urinary tract malignancies (bladder, upper urinary tract, prostate and kidney) were excluded. Patients with BPH, 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 BC, also developing in elderly men. Samples were checked for leucocytes, erythrocytes and nitrite (Bayer, multistix 10 SG). Additionally, 29 blood samples from patients with BC were included in the study.

Step 2. Urine samples collected 1 day before TUR of the recurrent tumour (pre-TUR urine; $n = 68$) were selected for analysis. After TUR, the stage and grade of the recurrent tumour were confirmed by an expert uropathologist. Control urine samples from non-BC patients ($n = 91$) were drawn from the Dutch Bladder Cancer Screening Study.

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

TABLE 1 Patient and tumour characteristics

	Age, years		Tumour stage, *n* (%)					Tumour grade, *n* (%)				
Characteristic	*N*	Mean (SE, range)	*N*	Ta	T1	T2	Tis	*N*	G0	G1	G2	G3
Step 1												
Tumour	37	69.2 (15.2, 26–86)	37	36 (97)	1 (3)			37		14 (38)	23 (62)	
Non-BC urine	46	54.1 (13.6, 26–74)										
Blood	29	59.3 (9.6, 40–80)										
Step 2												
BC-urine	68	65 (11, 34–85)	68	61 (90)	7 (10)			68		13 (19)	52 (77)	3 (4)
Non-BC urine	91	62 (6, 51–73)										
Step 3												
Follow-up urine	109	63 (11.6, 20–82)										
BC-urine			49	38 (78)	9 (18)		2 (4)	49		15 (31)	22 (45)	12 (24)
Step 4												
Follow-up urine	94	64 (11.1, 42–86)										
BC-urine			65	58 (89)	6 (9)	1 (2)		65	3 (5)	25 (38)	29 (45)	8 (12)

Non-BC urine, urine from patients without BC; BC-urine, urine from patients with BC; Follow-up urine, urine collected from patients with BC during follow-up (with and without recurrence).

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

DNA ISOLATION FROM PATIENT MATERIAL

Fresh tumourtissue

Tumour tissue was collected at 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. MI tumours and carcinoma *in situ* were excluded from analysis. Fresh tumour samples for DNA isolation were stored at –80 °C until DNA isolation. DNA was extracted using the DNeasy®blood and tissue kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol and analysed with the MS-MLPA assay.

Urine

Freshly voided urine samples (10–100 mL) were centrifuged for 10 min at 2000***g***. Cell pellets were washed twice with 10 mL PBS, followed by centrifugation for 10 min at 2000***g***. Pellets were re-suspended in 1 mL PBS, transferred to an Eppendorf vial and centrifuged for 5 min at 3000***g***. Supernatant was discarded and the cell pellet was stored at –20 °C until DNA isolation. DNA was extracted using the QIAamp Mini and Blood mini kit (Qiagen) 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) according to the manufacturer's protocol.

MS-MLPA

For fresh tumour tissue or urine-derived genomic DNA,50 ng was used for the MS-MLPA assays that were performed as described by Nygren *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. After 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 analysed 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 tumour suppressor genes with 15 reference probes and kit-002 contains 27 probes for tumour suppressor genes with 14 reference probes. Kit-001 and -002 contain probes for the same genes, but the probes recognise a different CpG-site.

STATISTICAL ANALYSIS

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 least absolute shrinkage and selection operator (LASSO) 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 (AUC).

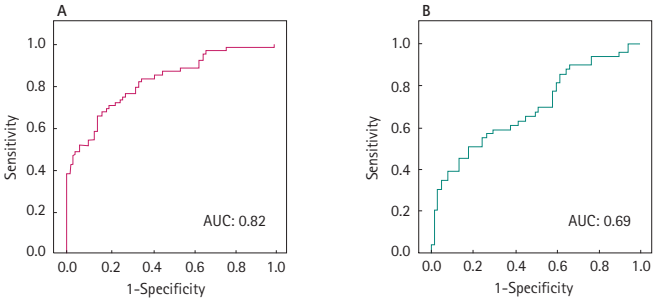
RESULTS

CONSTRUCTION OF THE BC MS-MLPA

To select genes for a BC-specific MS-MLPA assay, recurrent NMIBC tumours ($n = 37$) were analysed with the commercially available MS-MLPA kit-001 and -002 and methylated genes were selected to develop a BC-specific MS-MLPA (Fig. 1, Step 1). Urine samples of non-BC patients($n = 46$) and blood samples from patients with NMIBC ($n = 29$) were selected as controls. Patient and tumour characteristics are given in Table 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 tumour tissue compared with urine of non-BC patients. Methylation of *TP53* ($P = 0.034$), *MGMT* ($P = 0.031$), *PAX5* ($P < 0.001$), *CDH13* ($P < 0.001$), *TP73* ($P = 0.039$), *WT1* ($P < 0.001$), *ESR1* ($P < 0.001$), *MSH6* ($P = 0.005$), *RARB* ($P = 0.005$), and *CD44* ($P = 0.039$) from kit-002 was significantly higher in tumour tissue compared with 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 the above findings with the addition of six genes methylated in patients with BC 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 shown in Table 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 analysed the same available tumours and control non-BC urine samples with the newly developed BC MS-MLPA to validate the selection of probes. Results from the analysed non-BC urine samples ($n = 40$) and NMIBC tumour samples ($n = 34$) are given in Table 3. Significantly higher methylation of tumour tissue was reached for *TERT_b*($P < 0.001$), *ESR1_a* ($P < 0.001$), *EDNRB* ($P < 0.001$), *TERT_c* ($P = 0.001$) and *CDH13* ($P < 0.001$). 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 tumour tissue of patients with NMIBC.

*FIG. 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).*



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 (Fig. 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 vs 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 tumour and control and were combined into the final gene panel. This panel reached an AUC under the receiver operating characteristic (ROC) curve of 0.82 (Fig. 2A).

RECURRENCE RISK PROBABILITY INCREASES WITH STAGE AND GRADE

The selected gene panel was validated on an independent dataset of 49 pre-TUR and 60 control urine samples of recurrence-free patients with BC. The AUC for the gene panel reached 0.69 in this validation (Fig. 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 threshold (0.34), with the best sensitivity and specificity, was selected to indicate whether a test outcome was considered positive (detected recurrence) or negative (missed recurrence) (Fig. 3A). This resulted in a sensitivity and specificity of 31/49 (63.3%) and 35/69 (58.3%), respectively (Table 4). All high-grade tumours i.e. T1, grade 3 and carcinoma *in situ*, were detected and most were associated with a high recurrence risk probability. Missed tumours were of low risk potential, namely TaG1–2.

METHYLATION-BASED PROBABILITY, A PROMISING TOOL FOR THE DETECTION OF HIGH-RISK TUMOURS

Finally, we validated the selected threshold probability of 0.34 on an independent set of 65 pre-TUR urine samples and 29 urine samples from recurrence-free patients. The sensitivity and specificity were 47/65 (72.3%) and 16/29 (55.2%), respectively (Table 4). High-risk tumours were detected in 10 of 11 and mostly associated with a high recurrence risk probability. Mainly pTaG1–2 tumours were missed, together with one pT1G2 tumour (Fig. 3B).

DISCUSSION

Promoter methylation is a frequent finding in bladder tumours and previous research has indicated the possibility of methylation

TABLE 2 Probemix of the bladder cancer MS-MLPA assay

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-GACCGACATGTGGCTGTATTGGTGCAGCCCGCCAGGGT
154	TERT	CGCAGCGCGGGGACCCGGCGGCTT-TCCGCGCGCTGGTGGCCCAGTGCCTGGT
160	TERT	TCTGTGTCCTGCCTGAAGGAGCTGGT-GGCCCGAGTGCTGCAGAGGCTGTGCGAGCGCG
172	WIF1	TCTGTCTAAACGGGAACAGCCCTGGCT-GAGGGAGCTGCAGCGCAGCAGAGTATCTGACGGCGCC
178	CCM2	CTGGAATTGTCTCGCCATTTAAACGAGTAT-TCCTAAAAGGTGAAAAGAGTAGAGATAAGAAAGCCCATGAGAAGGT
193	RARB	CCGCCGGCTTGTGCGCTCGCT-GCCTGCCTCTCTGGCTGTCTGCTTTTGCAGGGCTGCT
202	MLH3	GCGACCTTGTTCTTCCTTTCCTTCCGA-GAGCTCGAGCAGAGAGGACTGTGATGAGACAGGATAACAG
211	TGFBR1	GGCGTTACAGTGTTTCTGCCACCTCT-GTACAAAAGACAATTTTACTTGTGTGACAGATGGGCTCTGCTTT
220	COL2A1	GCCTCTTCCTTCTACACAGGGTCCTT-CTGGAGACCAAGGTGCTTCTGGTCCTGCTGGTCCTTCT
229	PAH	CAGTGCCCTGGTTCCCAAGAA-CCATTCAAGAGCTGGACAGATTTGCCAATCAGATTCTCAG
238	CDKN2A	CTGGATCGGCCTCCGACCGTAAC-TATTCGGTGCGTTGGGCAGCGCCCCCGCCTCCAGCAGC
247	PTCH	GTGGACAGCTGGGAGGAAATGCTGAA-TAAGGCTGAGGTTGGTCATGGTTACATGGACCGCCCCT
259	CDKN2A	CCAGGGGCACCAGAGGCAGTAACCATG-CCCGCATAGATGCCGCGGAAGGTCCCTCAGA
265	SLC2A1	CTCTGGTCCCTCTCAGTGGCCATCTTTTCT-GTTGGGGGCATGATTGGCTCCTTCTCTGTGGGCCTTTTCGTTAACC
283	BCL2	GTGAAGCGGTCCCGTGGATAGAGA-TTCATGCCTGTGCCCGCGCGTGTGTGCGCGCGTG
292	PTEN	CACCGGAGCGGGCGCAGGAGA-GGCCTGCGGGGTGCGTCCCACTCACAGGGAT
301	ESR1	GCTCGCGTGTCGGCGGGACAT-GCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTCC
310	DBC1	CTGGAGGTTTGTTGAGCTCCTCTACT-TCCTGTTTATATGGGGCCGTATCTCAGTGCAGCCC
319	TIMP3	CATCGTGCTCCTGGGCAGCTGGA-GCCTGGGGGACTGGGGCGCCGAGGCGTGCACA
328	KRIT1	TGGTCTAGGAGCTCCAGACTACTCAAAAAT-CCAAATACCTAAACAGGAAAAATGGCAGAGAAGCATGAGCAGT
337	CDH1	CTATGAAGGAAGCGGTTCCGAAGCTGCTA-GTCTGAGCTCCCTGAACTCCTCAGAGTCAGACAAAGACCAGGAC
346	TNFRSF25	CTGAAGGCGGAACCACGACGGGCA-GAGAGCACGGAGCCGGGAAGCCCCTGGGCGCCCG
355	TGFBR1	GAGAATGTTGGTATGCCAATGGAGCA-GCTAGGCTTACAGCATTGCGGATTAAGAAAACATTATCGCA
364	DBC1	TCACTTCCTGACCATCGGAAGCATCCA-TCAGCACTGGGGCAATGACTGGGACCTGCAGA
375	ESR1	CCAGCCCGCCGTGTACAACTACCCCG-AGGGCGCCGCCTACGAGTTCAACGCCGCGGC
382	PTCH	GATAAGAGCTCCGGGGGGATTCTTCA-TGCACCAGTGTTTAGGGTACGTGCTTCCTAAGTAAATCCAAACATTG
391	CDKN2A	GCTCTTCCGCCAGCACCGGAGGAA-GAAAGAGGAGGGGCTGGCTGGTCACCAGAGGGTG
400	TIMP3	CCAGCGCTATATCACTCGGCCGCCCA-GGCAGCGGCGCAGAGCGGGCAGCAGGCAG
409	EDNRB	CCAAGTTTCCCACTGGCGCGCAA-ACTTGAGTTACTTTTGAGCGTGGATACTGGCGAAGAGGCTG
418	TERT	CCAGAGTTTCAGGCAGCGCTGCGTCCT-GCTGCGCACGTGGGAAGCCCTGGCCCCGGCC
427	APAF1	CCATTCATGCTTGTTTTGTTTTGGATTTTAGTAAGGACA-GTCCTGTGTGAAGGTGGAGTACCACAGAGGCCAGTTGTTTTTG
436	CDH13	GTTCTGTGCGTTCTCCTGTCCCAG-GTAGGGAAGAGGGGCTGCCGGGCGCGCTCTG
445	DBC1	GCAGTCACTCCTCTACTGTAATGAGAATGGGTT-TTGGGGAACCTTCCTGGAGAGCCAGCGGAGCTGCGTG
454	APC	CTCAGCTGTGTAATCCGCTGGATGCGGACC-AGGGCGCTCCCCATTCCCGTCGGGAGCCCGC
463	WIF1	CCTGCAAATAGAGCGAGAACAGAAGAGCGGGA-AGGGCTGGCGCGAGCGAGGTGCGAGCGAGGAG
472	CDH1	CTGAGGAGCGGAGCGGCCTGGAA-GCCTCGCGCGCTCCGGACCCCCCAGTGATGGGAGT
481	RARB	CCACATGTGCTTTTTCTGGAGTGGAAAAATACATA-AGTTATAAGGAATTTAACAGACAGAAAGGCGCACAGAGGAATTT

detection in urine. As methylation has been associated with bladder tumour stage, grade, recurrence and progression, detection of methylated tumour cells shed in urine might be a promising tool during the follow-up of patients with NMIBC [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 the clinical 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 tumours and that the recurrence risk increased with tumour stage and grade.

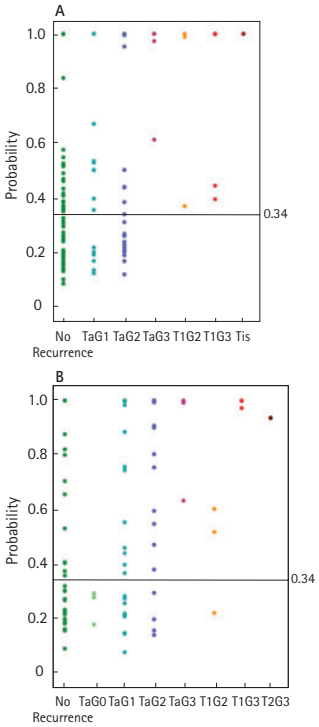
As voided urine does not contain many cells and there is a limited availability of clinical samples for molecular analyses, newly developed tests should be able to detect a low number of tumour cells and require small amounts of input DNA. The BC MS-MLPA assay is a sensitive method for detecting tumour cells and requires only 50 ng 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

Genes	Urine non-BC ($n = 40$) M	%M	Tumour tissue ($n = 34$) M	%M	P^*
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	<0.001
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	<0.001
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	0	23	67.6	<0.001
TERT_c†	0	0	8	23.5	0.001
CDH13†	0	0	9	26.5	<0.001
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

TABLE 3
Methylation frequencies for the BC MS-MLPA custom kit

**Chi-squared test (Bonferroni correction $\alpha = 0.002$); †Statistically significant methylated genes.*

FIG. 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.



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 bisulphite 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 tumours were low risk TaG1–2. Only one pT1G2 was missed. Thus, the methylation detection assay is able to detect recurrent and progressing tumours. Compared with previous studies the sensitivity of the present assay is relatively low [25,27,28,33]. Friedrich *et al.* [25] reported a sensitivity of 78% for the detection of methylation in urine samples associated with a primary tumour collected before radical cystectomy (22/37 ≥ pT2). Although size was not recorded in their publication, primary tumours and tumours selected for radical cystectomy are often large in size, have a higher grade and more genetic aberrations than recurrences. Accordingly, Catto *et al.* [18] reported that methylation levels increase with advanced tumour stage, possibly leading to an easier detection of these tumours. Dulaimi *et al.* [27] 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 of 45 tumours were classified as MI. Similarly, recently Renard *et al.* [29] developed a two-gene panel (*TWIST1* and *NID2*) to identify tumours in voided urine samples from 466 patients with primary BC (sensitivity 90%; specificity 93%). Notably, most studies use urine from patients without BC as a control group, but we think 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 tumour compared with control patients without a history of BC. The selection of the present gene panel was based on recurrent NMIBC tumours and the test was validated on a subset of urine samples from patients with BC with no recurrence at the time of urine collection.

Recurrent tumours (mainly TaG1–2, and one T1G2) were not detected in 32% of the cases (18/49+18/65) by the methylation assay (Table 4). Possibly, tumour cells were absent at the time of urine collection or the number of urinary tumour 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 showed that not all tumours are methylated, hence stratification according to methylation status of the primary tumour

TABLE 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, no. methylated (>0.34)/ total (%)		
All cancers	31/49 (63)	47/65
Ta	20/38 (53)	41/58
T1	9/9	5/6
Tis	2/2	1/1
Low grade	19/37 (51)	39/57 (68)
High grade	12/12	8/8
Specificity rate of gene panel, no. not methylated (>0.34)/ total (%)		
Recurrence-free patients with BC	35/60 (58)	16/29 (55)

could improve the sensitivity of the test (unpublished data).

'False'-positive urine samples were detected in 33% (25/56 + 13/60) of the cases (Table 4). Firstly, BC appears to arise from a field of change that affects not only macroscopically visible tumours, but also the area surrounding the tumour [34]. It might be possible that even though no tumour is detected cystoscopically, urothelial cells in the surrounding area of the resected tumour contain epigenetically changed cells. Secondly, multiple studies have shown residual tumour in 35–81% at the same site of the initial resection at the first follow-up cystoscopy or at deep resection of the tumour base [35–37]. Thirdly, it is known that cystoscopy does not detect all tumours and recent studies show that the sensitivity of the currently used white light cystoscopy varies between 68–83%. These studies also show that blue light fluorescence cystoscopy has superior sensitivity and studies on microsatellite analysis show an increased sensitivity of cystoscopy when the urologist was aware of a positive urine test outcome [5–7,38].

Most biomarkers have a chosen threshold 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 inreduced accuracy of the developed test. We designed a logistic regression model, based on raw methylation input data, to predict the probability of a recurrent tumour. A threshold probability of 0.34 was selected for having the highest sensitivity and specificity.

Limitations of the present study are the absence of tumour material for the test and validation subsets. Possibly, methylation was absent in the tumour, 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.

In conclusion, this is the first study to develop a methylation assay specific for the detection of NMIBC recurrent tumours 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.

ACKNOWLEDGEMENTS

This study was funded by a grant from the Koningin Wilhelmina Cancer Foundation (KWF): 2006-3672 and by the European Union (FP7, project 201663, UROMOL). The authors wish to thank all patients for their contribution and Lucie Kompier for sample collection.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **(NMI)(MI)BC**, (non-muscle-invasive)(muscle-invasive) bladder cancer; **MS-MLPA**, methylation-specific multiplex ligation-dependent probe amplification; **LASSO**, least absolute shrinkage and selection operator; **AUC**, area under the curve; **TUR**, transurethral resection; **ROC**, receiver operating characteristic.

CHAPTER 7

Combinations of Urinary Biomarkers for Surveillance of Patients with Incident Nonmuscle Invasive Bladder Cancer: The European FP7 UROMOL Project

J Urol. 2013 May :189(5):1945-51

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Combinations of Urinary Biomarkers for Surveillance of Patients with Incident Nonmuscle Invasive Bladder Cancer: The European FP7 UROMOL Project

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Purpose: We determined a combination of markers with optimal sensitivity to detect recurrence in voided urine after resection of an incident low grade, nonmuscle invasive bladder tumor.

Materials and Methods: A total of 136 patients with G1/G2 nonmuscle invasive bladder tumor were included in the study at transurethral resection of the incident tumor. At least 3 followup urine samples were required for patient selection. DNA was extracted from the incident tumor and cell pellets of subsequently collected urine samples. We performed *FGFR3*, *PIK3CA* and *RAS* mutation analysis, and microsatellite and methylation analysis on tissue and urine DNA samples.

Results: We obtained 716 urine samples. The 136 patients experienced a total of 552 recurrences during a median 3-year followup. Sensitivity for detecting a recurrent tumor varied between 66% and 68% for the molecular tests after patient stratification based on tumor DNA analysis. A combination of markers increased sensitivity but decreased the number of patients eligible for a certain test combination. Combining urine cytology with *FGFR3* analysis without stratifying for *FGFR3* status of the incident tumor increased sensitivity from 56% to 76%.

Conclusions: A combination of markers increased the percentage of patients eligible for urine based followup and the sensitivity of recurrence detection. Adding *FGFR3* analysis to urine cytology could be valuable for noninvasive followup of patients with nonmuscle invasive bladder cancer.

Key Words: urinary bladder; urinary bladder neoplasms; neoplasm recurrence; biological markers; receptor, fibroblast growth factor, type 3

Cystoscopy is the gold standard for the followup of patients with NMIBC and cystoscopy frequency is determined by the risk of recurrence and progression according to European Association of Urology guidelines.[1] However, cystoscopy is a time-consuming procedure that causes physical discomfort in patients and sensitivity has been estimated to vary from 68% to 83%.[2,3]

After resection, 1 or multiple recurrences develop in up to 70% of patients with NMIBC. Thus, patients frequently undergo transurethral resection of the tumor and together with multiple cystoscopies this makes bladder cancer one of the most expensive tumor types.[4,5] In addition to cystoscopy, urine cytology is performed but it has low sensitivity for detecting

low grade tumors.[6] Nevertheless, cytology is still used as a reference in the absence of highly sensitive urinary biomarkers.

To date multiple studies have been performed, usually investigating 1 type of urinary maker. Various markers have proved to be more sensitive than cytology but to our knowledge no single marker has attained sensitivity high enough to safely decrease the number of cystoscopies. A major problem with many studies that test biomarkers for urine diagnosis is that investigated urine samples are often derived from patients with incident tumors, which are mostly larger than recurrent tumors and also of higher grade. This results in high sensitivity and subsequently marker sensitivity decreases considerably when tested on urine samples from patients with a recurrent tumor.[7] Patients eligible for urine based surveillance typically present with G1 or G2 NMIBC. The risk of progression in these patients is low but the risk of multiple recurrences is high. Previous studies demonstrated the role of hypermethylation, leading to transcriptional silencing of tumor suppressor genes, and aberrant methylation was detected in the voided urine of patients with bladder cancer, which appears to be more sensitive than urine cytology.[8–13] We recently developed a methylation assay, ie the bladder cancer specific MS-MLPA assay. This methylation assay was specifically developed to detect recurrent NMIBC in voided urine.[14]

The use of MA to detect LOH in voided urine samples was also reported in multiple studies.[15–17] van der Aa et al observed that the positive predictive value of MA was higher in patients without *FGFR3* mutations.[15] Mutations in *FGFR3*, leading to constitutive activation of the RAS-mitogen activated protein kinase pathway, have been found in 70% of nonmuscle invasive bladder tumors and are associated with an overall favorable prognosis.[18,19] *RAS* mutations (*KRAS*, *HRAS* and *NRAS*), which have been found in 13% of bladder tumors of all stages and grades, are mutually exclusive with *FGFR3* mutations.[20] Previous research revealed *PIK3CA* mutations in 13% to 27% of bladder tumors, while *PIK3CA* mutations mostly co-occurred with *FGFR3* mutations.[21]

Recently, we reported that urinary *FGFR3* mutation analysis in patients with an incident *FGFR3* mutant tumor was a suitable assay to detect recurrence.[22] We also found that 88% of patients with low grade NMIBC 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 detect a mutation or a methylated gene. Thus, they represent a single assay. It is not possible to multiplex the MA, making this assay more labor intensive. Further, developments such as next generation sequencing would facilitate combining assays. Therefore, it is worthwhile to test whether a combination of assays would be better than a single assay.

We determined a combination of markers with optimal sensitivity to detect recurrence in voided urine. Therefore, we used mutation analysis of *FGFR3*, *PIK3CA*, *HRAS*, *KRAS* and *NRAS* combined with methylation specific MLPA and MA assays to increase the percentage of patients who could be monitored by urinalysis. Results were compared to those of urine cytology in a large, retrospective longitudinal cohort. This study was part of the European FP7 UROMOL project.

MATERIALS 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, Aarhus University Hospital at transurethral resection of the incident tumor. Patients with a history of carcinoma in situ were excluded from analysis. Urine (10 to 50 ml) was collected at regular followup visits. Urine specimens were collected immediately before cystoscopy. Cells were sedimented by centrifugation and frozen at −80C. Tumor biopsies were stored at −80C immediately after tumor resection. Cystoscopic examination coincided with urine collection for molecular analysis. The availability of at least 3 followup urine samples was a prerequisite for study inclusion. Recurrence was defined as histologically proven tumor. Progression was defined as progression to muscle invasive disease. Informed written consent was obtained from all patients. 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 the DNeasy® Blood and Tissue Kit. DNA was extracted from urine cell pellets using the QIAamp® Mini and Blood Mini Kit.

Mutation analysis for *FGFR3, PIK3CA*, and *RAS* was performed as described previously.[22,23,25] LOH was detected by MA using primers for 12 polymorphic microsatellite markers localized on chromosomes 8 to 11 and 17 (van Tilborg et al, unpublished data). MS-MLPA was used to detect methylation, as described by Nygren et al.[26] This MS-MLPA was specifically developed to detect recurrent bladder cancer.[14] The supplementary file (http://jurology.com/) shows all details of primers, probes and conditions.

Statistical Analysis

PASW 17 (SPSS®) was used for data analysis. Statistical differences were considered significant at $p < 0.05$. The chi-square and Fisher exact tests were used to determine relationships between different variables.

RESULTS

Patient and Tumor Characteristics

A total of 136 cases of incident G1 or G2 NMIBC were included in molecular analyses. Patients with G3 incident tumors were excluded due to a high risk of progression. Included patients had a total of 552 recurrences during a median 3-year followup. Table 1 lists patient and incident tumor characteristics. Four patients had progression to muscle invasive disease. DNA was available from the incident tumor in 93 of 136 patients and from the tumor at the first visit in 22. Tumors were analyzed for mutations in *FGFR3*, *PIK3CA* and *RAS (HRAS, KRAS* and *NRAS*), and for LOH and methylation. *FGFR3*, *PIK3CA* and *RAS* mutations were detected in 78 of 115 (68%), 20 of 111 (18%) and 10 of 110 cases (9%), respectively. *PIK3CA* and *FGFR3* mutations occurred simultaneously in 16 of 20 cases (80%), while *FGFR3* and *RAS* mutation were overall mutually exclusive since only 1 patient harbored the 2 mutations. When combined, 91 of 115 patients (79%) had a mutation in *FGFR3, PIK3CA* or *RAS* (fig. 1). LOH was detected in 72 of 114 patients (63%) and methylation was detected in 88 of 90 (98%). *FGFR3* mutations or LOH were detected in 81% of incident tumors.

Table 1. *Clinical and molecular characteristics of 136 patients and incident tumors*

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

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

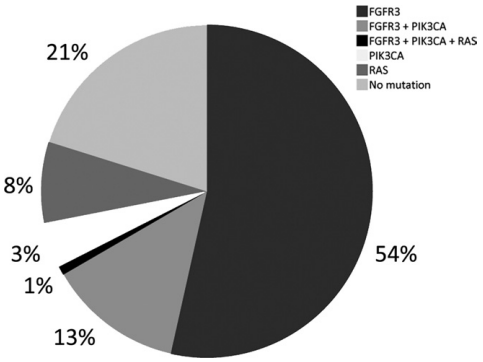

Figure 1. Mutation status of 115 incident tumors. Of patients 79% had activating mutation in at least 1 tested oncogene and 21% had no mutation.

During followup, 552 histologically proven recurrences were detected, including mainly stage Ta (92%), G1/2 (82%) and solitary tumors (67%) (table 2). Overall, recurrent tumors in patients with a *FGFR3* mutant incident tumor were of lower stage and grade than recurrent tumors in patients with a *FGFR3* wild-type incident tumor ($p = 0.001$ and 0.003, respectively, fig. 2). No upper tract recurrence was detected.

Performance of Single and Combined Urine Tests to Detect Recurrent Tumors

Based on the molecular characteristics of the incident tumors, a combination of *FGFR3* mutation analysis with another mutation assay or MA, or MS-MLPA increased the percentage of patients suitable for urine based followup. Of all urine samples 99% could be analyzed for *FGFR3* mutations. Due to the limited amount of DNA in some urine samples,

Table 2. *Clinical characteristics of 552 recurrent tumors*

	No. Tumors (%)
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	370 (67)
Multiple	182 (33)
Tumor size (cm):	
Less than 3	519 (94)
Greater than 3	20 (4)
Unknown	13 (2)

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

only 70% and 63% could be analyzed for LOH and methylation, respectively. Enough DNA remained to analyze *RAS* and *PIK3CA* mutations in 20% of all urine samples.

For each single and combined assay we determined the sensitivity of detecting a recurrent tumor in voided urine. Patients were stratified by the molecular status of the incident tumor and analyses were done in each subgroup. We did not calculate the sensitivity of *PIK3CA* or *RAS* mutation analysis in recurrent tumors separately because of the few urine samples available for analysis. For MA we also calculated sensitivity using only the 5 markers for loss on chromosome 9. Figure 3 shows the sensitivity of all single and combined tests. Sensitivity for detecting recurrence in patients with a *FGFR3* mutant incident tumor (stratified) was 66%. Adding *PIK3CA* and *RAS* assays to *FGFR3* mutation analysis increased the sensitivity for detecting recurrence 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%) to detect recurrence. For this combination patients were stratified based on an *FGFR3* mutation in the incident tumor and/or LOH in the incident tumor. This analysis was possible in 81% of patients. Since analysis with 12 MA markers requires a high amount of DNA, we also calculated the sensitivity of detecting recurrent tumors using only the 5 markers for loss on chromosome 9. The sensitivity of MA was then 64% compared to 67% for the analysis with all 12 markers. When combined with *FGFR3* analysis, sensitivity increased to 76%. For the *FGFR3*/cytology combination patients were not stratified based on the incident tumor. This combination attained 76% sensitivity.

Overall, the specificity for all single and combined tests was low, ranging from 34% for *FGFR3*/MA to 66% for *FGFR3* unstratified.

Molecular Analyses Were Superior to Urine Cytology for Detecting Low Stage, Low Grade Recurrence

The overall sensitivity of all single and combined molecular tests to detect recurrent tumors was higher than the sensitivity of urine cytology alone (fig. 3). Combining urine cytology with *FGFR3* analysis improved sensitivity from 56% to 76%. Table 3 shows the sensitivity of all tests stratified by stage and grade. The sensitivity of detecting high stage and high grade tumors was similar for the combined molecular tests and for urine cytology. However, for low stage and low grade tumors single and combined

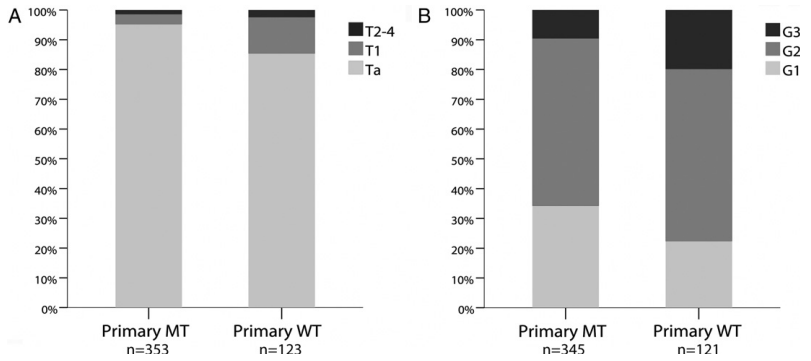

Figure 2. Stage (*A*) and grade (*B*) of 482 recurrent tumors. Patients were stratified by *FGFR3* mutation status of incident tumor. Six patients with Tx and 16 with Gx were not included in analysis. *MT*, mutant. *WT*, wild-type.

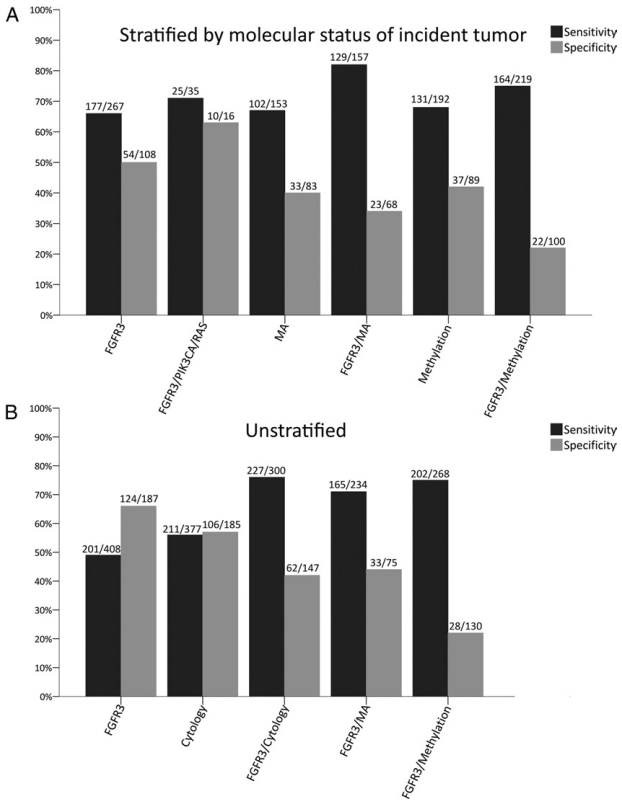

Figure 3. Sensitivity and specificity of all single and combined tests with (*A*) and without (*B*) patient stratification based on molecular status of incident tumor.

molecular tests were more sensitive than urine cytology. Adding *FGFR3* analysis to urine cytology improved the sensitivity of detecting low stage and low grade tumors, ie for Ta from 54% to 75% and for G1 from 33% to 66%.

DISCUSSION

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

The 66% sensitivity of *FGFR3* analysis for detecting concomitant recurrence in the urine of patients with a *FGFR3* MT incident tumor was comparable to that in previous reports.[22] Not all recurrent tumors were detected, which is mainly the result of the number of tumor cells that are below the analytical sensitivity of the assays. In a previous study we noted 100% and 75% sensitivity of the *FGFR3* assay for tumors larger and smaller than 1.5 cm, respectively.[27] Analyzing more than 1 urine sample could increase sensitivity. Combining *FGFR3* analysis with other molecular tests improved sensitivity. A

Table 3. *Test sensitivity stratified and not stratified by molecular status of incident tumor*

	No. Stage/Total No. (% sensitivity)				No. Grade/Total No. (% 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)	Not available	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	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)	Not available	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)

total of 79% of 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 3 mutation assays was 71%. The optimal combination for recurrence detection was *FGFR3*/MA based on stratification of the incident tumor for the presence of *FGFR3* mutations and/or LOH. Without patient stratification based on the molecular status of the incident tumor, the combination of *FGFR3*/cytology attained 76% sensitivity. Cytology is known to have low sensitivity for G1 (7% to 38%) and G2 (18% to 46%) tumors.[28] Thus, for surveillance the combination of cytology and the *FGFR3* mutation assay, which is more directed toward low grade/stage tumors, 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 develop in sufficient amounts in nonbladder 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 urinalysis, ie the urine test identifies recurrence earlier than cystoscopy.[22] For example, in 46 of the 54 study patients (85%) with false-positive *FGFR3* analysis a recurrent tumor was detected within 12 months. In addition, cystoscopy misses tumors since currently used white light cystoscopy is estimated to have 68% to 83% sensitivity.[2,3]

Clinical implementation of a molecular test depends not only on the performance of the marker but also on costs, specifically since bladder cancer is an expensive cancer. Mutation analysis is inexpensive with a consumable cost of less than $10 per sample, including DNA isolation. The tests can be performed at a standard laboratory and require only 5 ng DNA. Based on our findings in this retrospective study, 99% of urine samples would contain sufficient DNA to perform the *FGFR3, PIK3CA* and *RAS* mutation assays. Although MA is inexpensive, the test requires 12 individual polymerase chain reactions per sample. Also, analyzing the results is laborious and would need automation before implementation in routine clinical practice. Moreover, a substantial percentage of urine samples do not yield sufficient DNA to perform MA. However, the amount of markers could be decreased using only the 5 markers for chromosome 9. Markers in the MS-MLPA kit have high predictive value. Unfortunately, in our hands the test appeared not to be a suitable technology for introduction into clinical practice. The reason is that in many cases the polymerase chain reaction step failed and no product was apparent on the sequencer.

Study strengths include the analysis of a substantial number of incident tumors together with at least 3 followup 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 must be validated in a large prospective study, which is currently under way in the European FP7 UROMOL project.

CONCLUSIONS

To our knowledge we report the first development of an optimal combination of urine based tests to increase the sensitivity of recurrence detection. All tests had higher sensitivity than urine cytology for low stage and grade disease. Therefore, a combination of molecular tests or a combination of the *FGFR3* assay with cytology represents promising possibilities for following patients with low grade NMIBC. These findings will be validated in a large, prospective, multicenter study of the European FP7 UROMOL project.

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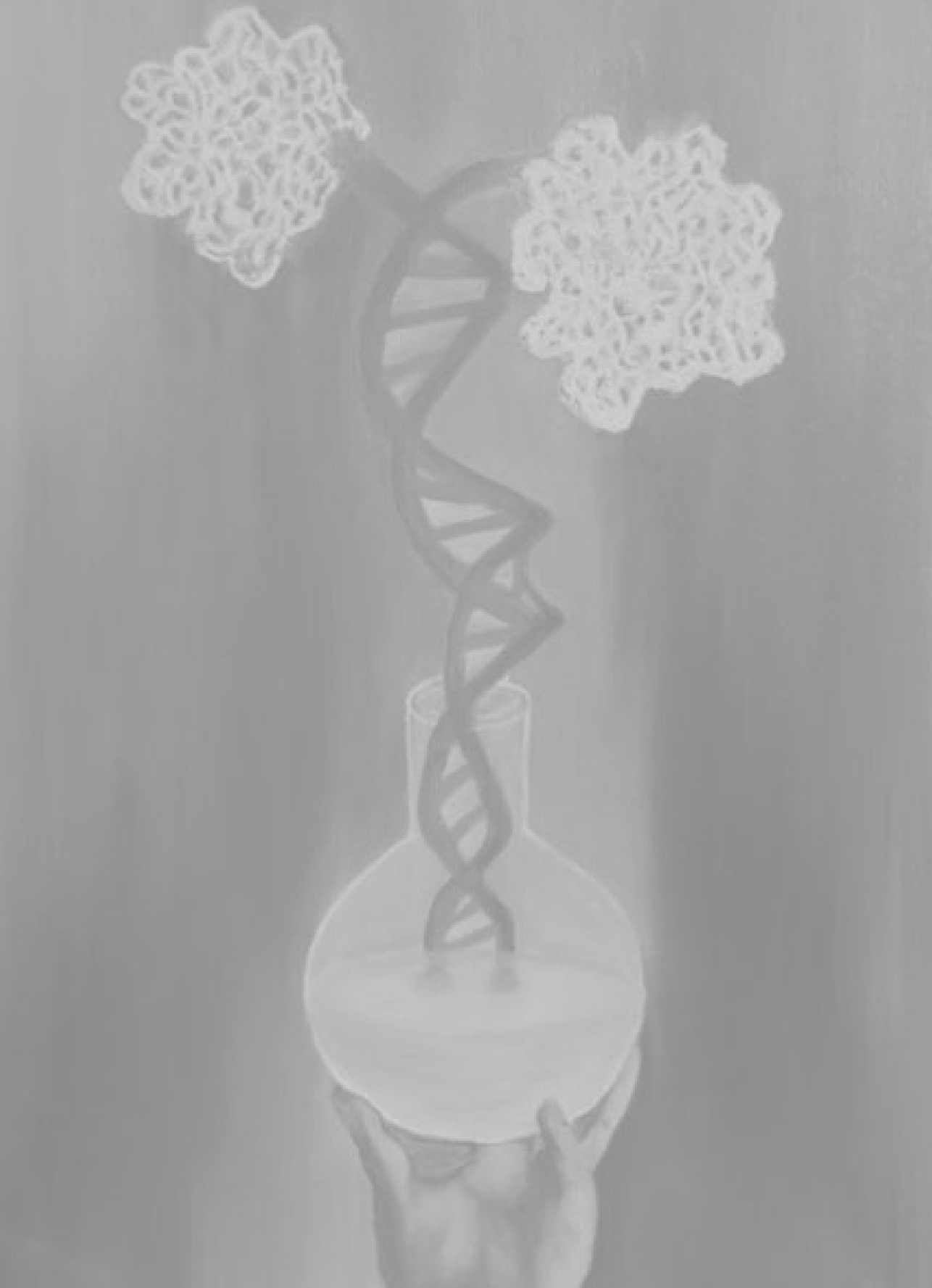

PART IV

SURVEILLANCE OF PATIENTS WITH HIGH-GRADE NMI-BC

Based on:

Chapter 8

Markers predicting response to bacillus Calmette-Guérin immunotherapy in high-risk bladder cancer patients: a systematic review

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Markers predicting response to bacillus Calmette-Guérin immunotherapy in high-risk bladder cancer patients: a systematic review

Eur Urol. 2012 Jan;61(1):128-45

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Review – Bladder Cancer

Markers Predicting Response to Bacillus Calmette-Guérin Immunotherapy in High-Risk Bladder Cancer Patients: A Systematic Review

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Article info

Article history:
Accepted September 27, 2011
Published online ahead of print on October 6, 2011

Keywords:
Bacillus Calmette-Guérin
BCG
Cytokine
Immune response
Immunotherapy
Interleukin
Intravesical instillation
Molecular marker
Non–muscle-invasive bladder cancer
Progression
Recurrence
Single nucleotide polymorphism
SNP
Survival

Abstract

Context: Currently, bacillus Calmette-Guérin (BCG) intravesical instillations are standard treatment for patients with high-grade non–muscle-invasive bladder cancer; however, no markers are available to predict BCG response.
Objective: To review the contemporary literature on markers predicting BCG response, to discuss the key issues concerning the identification of predictive markers, and to provide recommendations for further research studies.
Evidence acquisition: We performed a systematic review of the literature using PubMed and Embase databases in the period 1996–2010. The free-text search was extended by adding the following keywords: *recurrence, progression, survival, molecular marker, prognosis, TP53, Ki-67, RB, fibronectin, immunotherapy, cytokine, interleukin, natural killer, macrophage, PMN, polymorphism, SNP, single nucleotide polymorphism,* and *gene signature.*
Evidence synthesis: If thresholds for the detection of urinary interleukin (IL)-8, IL-18, and tumour necrosis factor apoptosis-inducing ligand levels are standardised, measurement of these cytokines holds promise in the assessment of BCG therapy outcome. Studies on immunohistochemical markers (ie, TP53, Ki-67, and retinoblastoma) display contradictory results, probably because of the small patient groups that were used and seem unsuitable to predict BCG response. Exploring combinations of protein levels might prove to be more helpful to establish the effect of BCG therapy. Single nucleotide polymorphisms, either in cytokines or in genes involved in DNA repair, need to be investigated in different ethnicities before their clinical relevance can be determined. Measurement of urinary IL-2 levels seems to be the most potent marker of all the clinical parameters reviewed.
Conclusions: IL-2 levels are currently the most promising predictive markers of BCG response. For future studies focusing on new biomarkers, it is essential to make more use of new biomedical techniques such as microRNA profiling and genomewide sequencing.

1. Introduction

Until now, intravesical bacillus Calmette-Guérin (BCG) instillations have proven to be the most successful adjuvant treatment for patients with intermediate- and high-risk non–muscle-invasive bladder cancer (NMIBC) [1]. Despite treatment, 30–50% of patients fail to respond, and 15% have progression to muscle-invasive disease (Fig. 1 shows the treatment protocol) [2–7]. Multiple factors could explain the high percentage of BCG failures. First, full compliance with the current protocol is affected by BCG-associated side effects. Second, high intra- and interobserver variability among pathologists, leading to incorrect histologic staging of tumours, could explain BCG failure [8,9]. Also, incomplete tumour resection, reported in 20–62% of cases, at restaging transurethral resection (TUR) could be the cause of refractory disease [10–13].

BCG response is currently determined by refractory disease after the first or second BCG induction course or by a recurrence during maintenance therapy. The only strong predictive marker used to identify patients for immediate cystectomy is refractory T1 or carcinoma in situ (CIS) disease after BCG induction [14]. Thus reliable markers to predict BCG response at an early phase are still needed to select the most effective treatment.

In this report, we review the literature on clinicopathologic, molecular, and inflammatory markers, perform critical analysis of the data, discuss the key issues in the identification of predictive markers, and provide recommendations for further research studies.

2. Evidence acquisition

A literature search was conducted using the PubMed and Embase databases for all published results from 1996 to 2010 on predictors of BCG response, which was measured by the outcomes of recurrence, progression, and survival. PubMed was searched using a combination of these text terms: *BCG* (or *bacillus Calmette-Guérin*) and *bladder cancer*. The free-text search was extended by adding these keywords: *recurrence, progression, survival, molecular marker, prognosis, TP53, Ki-67, RB, fibronectin, immunotherapy, cytokine, interleukin, natural killer, macrophage, PMN, polymorphism, SNP, single nucleotide polymorphism*, and *gene signature*. All articles were examined by two authors (TZ and AN), and papers related to our research question were selected.

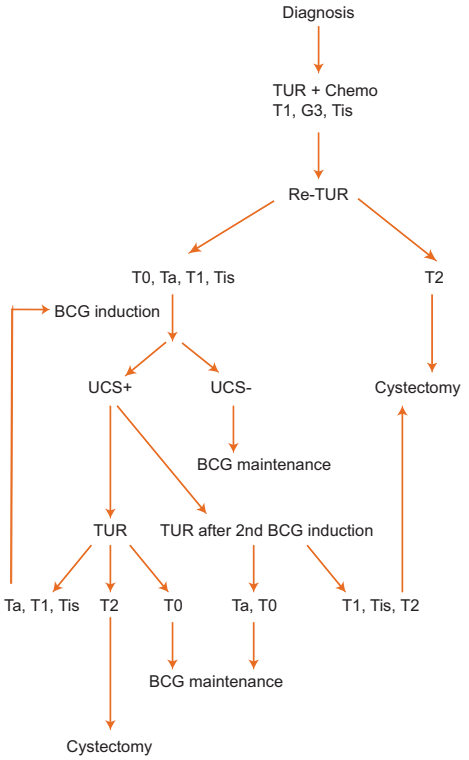

Fig. 1 – Bacillus Calmette-Guérin (BCG) treatment protocol. After diagnosis of all stages of non–muscle-invasive bladder cancer, patients receive an immediate postoperative instillation of chemotherapy (ie, mytomycin-C, epirubicin, or doxorubicin) within 6 h after transurethral resection (TUR). According to European Association of Urology guidelines, a re-TUR is performed within 6 wk when the resection has been incomplete (large and multiple tumours, no muscle in the specimen) or when an exophytic high-grade and/or T1 tumour has been detected. In case of a high-risk T2 tumour, immediate cystectomy is recommended. Patients with non–muscle-invasive tumours (Ta, T1, and carcinoma in situ [Tis]) receive adjuvant intravesical immunotherapy with BCG for 6 wk. If no recurrence is detected after treatment, BCG maintenance schedule is followed for at least 1 yr. In nonresponders, a second course of 6 weekly BCG instillations may be administered after the first one because 40–60% of these patients will respond to additional BCG treatment. In case of Tis BCG failure, a second induction cycle of BCG can be administered. If no or Ta lesions are visible after therapy, maintenance BCG schedule is initiated. Nonresponders (T1, Tis, or T2) receive radical cystectomy. Chemo = chemotherapy; UCS– = negative urethrocystoscopy; UCS+ = positive urethrocystoscopy.

3. Evidence synthesis

3.1. Types of markers

The effect of BCG treatment can be assessed at different stages (Fig. 2). Starting from a patient's diagnosis, early "pretreatment markers" in blood or urine may be examined (ie, clinicopathologic variables, single nucleotide polymorphisms [SNPs] of the host in nucleotide excision repair, and cytokine genes and/or urinary cytokine levels). The advantage of such an approach is early stratification of BCG nonresponders (BCG-NRs) who are at a high risk of progression and the selection of patients for the optimal treatment without any time delay. At (restaging) TUR, blood, urine, and tumour tissue can be used for analysis (ie, immunohistochemical detection of TP53, Ki-67, retinoblastoma, ezrin, and/or gene expression profile of the tumour).

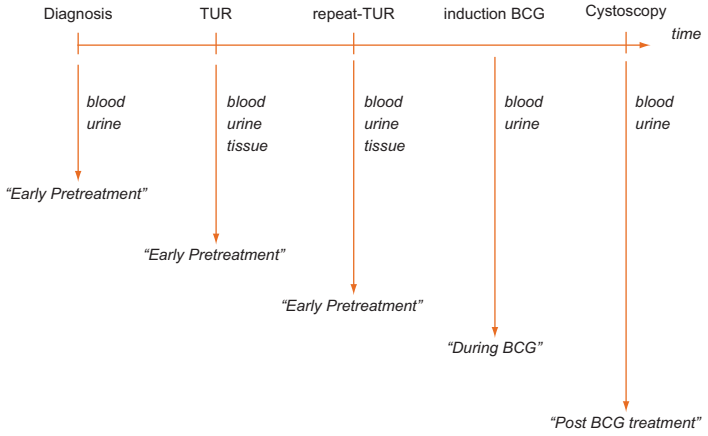

Fig. 2 – Biomarkers from diagnosis to treatment. Markers to evaluate bacillus Calmette-Guérin (BCG) effectiveness can be measured at different stages during treatment. "Early pretreatment" markers may include the detection of single nucleotide polymorphisms in blood to assess a patient's risk of progression, levels of inflammatory cytokines in urine can serve as "During BCG" markers to estimate the effectiveness of the immune response, and markers derived from the residual tumour in urine can be used as "Post BCG" indicators to detect the response to therapy.

During induction BCG, inflammatory markers can be measured in urine or blood to investigate the effectiveness of the inflammatory response; these can be described as "during-BCG markers" (ie, levels of dendritic cells, tumour-associated macrophages, interleukin [IL]-2, IL-8, IL-18, polymorphonuclear neutrophil [PMN] granulocytes, and tumour necrosis factor apoptosis-inducing ligand [TRAIL]).

Late "post-BCG markers" can be assessed in urine or blood to determine the presence of residual disease after treatment, but they seem less suitable to predict BCG response. To date, research on BCG therapy response has mainly focused on four groups of markers: clinicopathologic, inflammatory, cell cycle, and gene polymorphisms. We aim to investigate the clinical applicability of these markers.

3.2. *Clinicopathologic markers*

In general, factors assessing patient and tumour characteristics (ie, clinicopathologic parameters) hold valuable information for clinicians about treatment modalities and patient prognosis. Table 1 lists the most relevant studies on these markers predicting BCG response.

Patients presenting with a tumour at the first cystoscopy had a much higher risk of recurrence and progression than those who were tumour free [15–17]. In addition, history of bladder cancer, older age, concomitant CIS, multiplicity, and high-grade tumours all correlated with a significantly higher frequency of recurrences and progression in multivariate analysis [17,18]. Several papers reported that tumour size was associated with a poor prognosis; however, others did not find this correlation [17–19]. Although claims have been made in the past that patients with local or systemic BCG toxicity have a better treatment outcome, Sylvester and colleagues did not find this correlation [20]. Lastly, Boorjian et al. and Herr found that older age at diagnosis was associated with high-risk tumours, an increased recurrence risk, and a shorter cancer-free survival time [21,22].

In summary, several studies with large patient cohorts have been performed, but in most cases the variables that were significant in some studies were not significant in others. The discrepant findings regarding the predictive value of traditional pathologic markers in BCG response is also underlined by the results of an exhaustive review of the literature by Saint et al, covering 25 yr of research [23]. None of the host or tumour characteristics examined by the different groups in the review could be identified as independent predictive markers in all studies. Clinicopathologic variables might lose their informative potential in BCG-treated patients because the interpretation of pathologic samples is subject to major variability, and this tumour population was already selected for its aggressive potential.

Importantly, restaging TUR, which has only been implemented in the clinic regularly in recent years, allows for a better selection of patients for BCG therapy [14] and improves the initial response rate to BCG treatment [13]. This development causes a bias in the clinical data when comparing earlier studies with recent ones. However, only three papers in our literature search applied restaging TUR in their studies, and we have highlighted these papers with an asterisk in the tables. According to current European Association of Urology guidelines, recommendations in the treatment of high-risk tumours include a second TUR after 4–6 wk. Considering its advantages, it should be common practice in future studies.

Table 1 – Clinicopathologic markers to predict bacillus Calmette-Guérin response in non–muscle-invasive bladder cancer

Study	Patients	Clinical variables	Median follow-up, mo	End point	Results
Fernandez-Gomez et al. [17]	$n = 1062$	Gender/age	69	Time to first recurrence	Tumour multiplicity ($p = 0.001$), recurrent tumours ($p < 0.0001$), female gender ($p = 0.001$), high age ($p = 0.03$), and high grade ($p = 0.03$)
	Ta: 251	Primary vs recurrent tumour			
	T1: 1001	Tumour multiplicity		Progression to muscle-invasive disease	A recurrent tumour ($p < 0.0001$), high age ($p = 0.023$), high grade ($p < 0.0001$), and stage T1 ($p = 0.002$)
	Tis: 44	Tumour size			
	Concomitant CIS: 134	Tumour stage/grade Associated CIS			
Takashi et al. [18]	$n = 146$	Gender/age	64.7	Five-year recurrence-free survival	History of bladder cancer ($p = 0.017$), recurrence frequency down in patients without CIS ($p < 0.0001$)
	With CIS:	Primary vs recurrent tumour			
	Ta: 19 and TI: 15	Tumour multiplicity		Five-year progression-free survival	History of bladder cancer ($p = 0.002$), concomitant CIS ($p = 0.005$)
	Without CIS:	Tumour size			
	Ta: 89	Tumour stage/grade		Five-year overall survival	Age >70 yr ($p = 0.0006$), history of bladder cancer ($p = 0.036$), concomitant CIS ($p = 0.02$)
	T1: 23	Associated CIS BCG dose			
Andius et al. [15]	$n = 236$	Gender/age	44	Time to recurrence	Maintenance therapy ($p < 0.001$), negative first cystoscopy ($p < 0.001$)
	Ta: 169	No. of TURs before BCG		Time to progression	Tumour at first cystoscopy ($p < 0.001$), less than six BCG instillations ($p = 0.013$), grade ($p = 0.003$), history of upper tract tumour ($p = 0.023$)
	T1: 67	Accumulated No. of tumours before BCG			
		Length of tumour history before BCG Treatment year BCG strain Tumour stage/grade Previous upper tract tumour Six or fewer induction instillations Recurrence at first cystoscopy Maintenance BCG		Time to local failure	More than six BCG instillations ($p = 0.002$)
Andius et al. [16]	$n = 173$	Gender/age	72	Time to recurrence	Total no. of instillations ($p = 0.0001$), maintenance BCG ($p = 0.0014$), age ($p = 0.0014$)
	CIS: 173	No. of TURs before BCG		Time to progression	Negative first cystoscopy ($p < 0.001$)
		Accumulated No. of tumours before BCG			In patients with a negative first cystoscopy, no. of previous TURBs ($p = 0.02$)
		Length of tumour history before BCG		Time to local failure	Negative first cystoscopy ($p < 0.0001$)
		Treatment year		Time to death	Negative first cystoscopy ($p < 0.0002$)
		BCG strain			In patients with a negative first cystoscopy, patient age ($p < 0.0001$), less than six initial instillations ($p = 0.001$)
		Tumour stage/grade Previous upper tract tumour Six or fewer induction instillations Recurrence at first cystoscopy Maintenance BCG			
Lopez-Beltran et al. [19]	$n = 51$	Age	63.8	Disease-free survival	Tumour size ($p = 0.0034$)
	T1G3: 51	Gender		Progression-free survival	Tumour size ($p = 0.0001$)
		Tumour size		Overall survival	Tumour size ($p = 0.0296$), molecular markers (multivariate analyses)

Table 1 (*Continued*)

Study	Patients	Clinical variables	Median follow-up, mo	End point	Results
Boorjian et al. [21]*	n = 1021	Age	>60	Time to recurrence	High grade (p = 0.02)
	Ta: 612	Gender		Time to progression	Tumour stage (T1 vs Ta $p < 0.001$), grade (high vs low $p < 0.001$)
	T1: 409 Concomitant CIS: 629	Tumour grade/stage CIS			
		Hormonal status of woman (>50 yr of age)			
Herr [22]*	n = 805	Age	>24 (78% >60)	Recurrence	Age >70 yr associated with increased recurrence risk (p = 0.03)
	Ta: 396	Gender		Cancer-free survival time	Patients <70 yr of age (p = 0.005)
	T1: 409 Concomitant CIS: 629	Tumour stage/grade CIS			
		Tumour multiplicity Primary vs recurrent tumour Restaging TUR pathology Prior BCG			

Tis/CIS = carcinoma in situ; BCG = bacillus Calmette-Guérin; TUR = transurethral resection; TURB = transurethral resection of the bladder.
* Restaging TUR has been implemented in study.

3.3. *Inflammatory markers*

3.3.1. *Bacillus Calmette-Guérin–induced inflammatory response*

Current thoughts on the BCG-induced inflammatory response are that BCG-induced antitumour activity is part of a multistep process, ultimately leading to tumour eradication. How this course of action precisely works is still unknown, but different steps crucial to BCG activity have been identified and need to be discussed in relation to inflammatory markers (Fig. 3). Administered BCG binds to fibronectin (FN), exposed at the luminal surface of the tumour resection site, to form FN-BCG complexes that are internalised by normal urothelial cells and tumour cells [24–27]. Kavoussi et al. demonstrated that adherence of BCG to the bladder wall was inhibited by anti-FN antibodies in a murine bladder [24]. Boorjian et al. showed that patients treated with a fibrin clot inhibitor during BCG therapy had a higher risk of recurrence and progression to open surgery afterwards, underlining the importance of FN in BCG effectiveness [28]. After the cell processes BCG, a complex of BCG antigens and the major histocompatibility complex II is formed and expressed at the cell surface of normal urothelial cells, tumour cells, and antigen-presenting cells. This complex is recognised by $CD4^+$ T cells, leading mainly to a T helper-1 (Th-1) response and production of IL-2, IL-12, interferon (IFN)-γ, and tumour necrosis factor (TNF)-β. This leads to recruitment and activation of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), causing a massive immune response and eradication of tumour cells [29–31]. Additionally, a less favourable Th-2 response is induced together with production of IL-4, IL-5, IL-6, and IL-10. An influx of macrophages and large numbers of polymorphonuclear neutrophil (PMN) granulocytes has also been demonstrated [32–34]. The released leukocytes by this immunologic cascade are also secreted in the urine, and Saint et al. found a correlation between high levels of white blood cells (ie, leukocyturia) and a positive response to BCG therapy [35]. A word of caution might be that leukocyturia is a very aspecific marker because several clinical conditions, such as urinary tract infection in women or prostatitis, can cause elevated levels of leukocytes in the urine.

Several of the molecules and inflammatory cells discussed here have been studied as potential markers of BCG response (Table 2) and are reviewed in the following sections.

3.3.2. *Dendritic cells and tumour-infiltrating macrophages*

A high number of urinary dendritic cells (DCs) seem to have a positive effect on the outcome of BCG treatment [36]. Along the same lines, recurrence risk decreases with high RNA expression of antigen-presenting molecules in normal urothelial cells [37]. In patients with a weak initial immune response (which could be explained by a switch from a favourable Th-1 response, caused by only a few instillations, to a less favourable Th-2 response, caused by BCG maintenance), determined by low levels of $CD83^+$ tumour-infiltrating DCs, maintenance BCG proved to be highly effective by activating immune cells [38]. Although extensive infiltration of tumour-infiltrating macrophages (TAMs) has been reported to correlate with good prognosis in various types of cancer [39,40], in the biologic context of the bladder, high numbers of TAMs seem to have a negative role in BCG response [34,38], underlining the important role of the tumour microenvironment in BCG effectiveness [41–43].

Because the previously mentioned reports are single studies with small patient cohorts, their findings need to be validated in larger patient groups before tissue or urinary levels of DCs or TAMs can be applied in the clinic.

3.3.3. *Cytokines*

3.3.3.1. Interleukin-2. IL-2, a cytokine that is part of the Th-1 response, is secreted by activated $CD4^+$ cells and stimulates the growth, differentiation, and survival of CTLs. Several

Table 2 – Immunologic markers to predict bacillus Calmette-Guérin response in non–muscle-invasive bladder cancer patients

Study	Type of patient, No.	Type of marker	Sample type	Time of collection	BCG	Median follow-up, mo	End point	Results
BCG antigen- presenting markers								
Saint et al. [35]	$n = 72$ Multif or recurrent pap TCC: 2 Ta/T1 + grade 3 CIS: 9 Ta/T1 G3: 24 Ta/T1 G1: 37 and 2	Leukocytes	Urine	3 d after each instillation	6 weekly 81 mg ImmuCyst Maintenance 3 weekly	24	Recurrence Progr Adverse events	In multivariate analysis, leukocyturia only predictive factor for recurrence ($p = 0.009$) Leukocyturia only factor predicting adverse events ($p < 0.0001$)
Beatty et al, [36]	$n = 12$, NMIBC	DCs: CD45+, HLA-DR+	Urine	At first BCG At sixth BCG	6 weekly	10	Recurrence	Lower percentage of DCs in patients with a recurrence ($p = 0.14$) Higher percentage of DCs in patients without a recurrence ($p = 0.14$)
Ayari et al. [38]	$n = 66$, high risk Previous recurrence <6 mo Stage pT1 Grade 3 Diffuse Tis Size >3 cm	TIDC: CD83+ TAM: CD68+	FFPE tissue	Before BCG	6 weekly 3 weekly maintenance (36 mo)	26	Recurrence	TIDC levels not associated with recurrence ($p = 0.210$) One cycle of BCG maintenance and high TIDC levels = increased recurrence risk ($p = 0.045$) Maintenance BCG associated with lower recurrence risk in TIDC low ($p = 0.002$) High numbers of TAM equalled increased recurrence risk ($p = 0.013$)
Takayama et al. [34]	$n = 41$ CIS	CD68	FFPE tissue	Before BCG	80 mg Tokyo 172 81 mg Connaught 6 weekly	52.8	Recurrence	High TAM in tumour area (TAM-c) = decreased recurrence-free survival ($p = 0.0002$) TAM count in lamina propria (TAM-I) not related to recurrence-free survival
Videira et al. [37]	$n = 59$, NMIBC Group 1: recurrence-free <12 mo >TUR (28) Group 2: recurrence <12 mo >TUR (13) Group 3: recurrence at study entry (18)	CD1a/b/c/d/e MHC-I MIP-1α MCP-1/2 IP10 MIG	Tumour tissue Normal urothelium	At TURB 3 mo after TUR	6 weekly 3 weekly maintenance (12 mo)	12	Recurrence	BCG treatment increased expression of CD1a ($p < 0.005$), CD1b ($p < 0.00$) CD1c ($p = 0.03$), CD1e ($p = 0.007$), MHC-1 ($p < 0.0001$) MIG ($p < 0.0001$) and IP10 ($p < 0.0001$) in recurrence-free patients
Cytokines IL-2								
Saint et al. [44]	$n = 37$	IL-2	Urine	Before each weekly BCG	150 mg Pasteur	29	Recurrence	Higher recurrence risk when urinary IL-2 <27pg/μmol creatinine ($p = 0.0009$)

Table 2 (*Continued*)

Study	Type of patient, No.	Type of marker	Sample type	Time of collection	BCG	Median follow-up, mo	End point	Results
	Tis: 3 Ta/T1 and G3 + CIS: 3 Ta/T1 and G3: 8 Ta/T1 and G1/G2: 23 Healthy controls: 13	IFN-γ IL-10	Blood (controls)	6–8 h after BCG	6 weekly			Urinary IL-10 and IFN-γ no impact on recurrence risk or progr
Watanabe et al. [45]	*n* = 20	IL-1	Urine	At first BCG	80 mg Tokyo 172	46.9	Residual disease	Increase in IL-2, IL-6, IL-8, IL-10 TNF-β in responders ($p < 0.05$)
	Tis: 4 Secondary CIS: 11 Concurrent CIS: 5	IL-2 IL-6 IL-8 IL-10 IL-12 IFN-γ TNF-β		At eighth BCG Every 2 h	8 weekly		Recurrence	IL-2 independent prognostic factor for responders ($p = 0.003$)
De Reijke et al. [46]	*n* = 23 High risk: 19 Low risk: 4	IL-2	Urine	After each weekly BCG	$5 \times 10^{-}5$ CFU Connaught 6 weekly		Recurrence	High IL-2 after BCG 1 = increased recurrence-free survival ($p = 0.003$)
Saint et al. [47]	*n* = 39 Tis: 1 Ta/T1 G3 and CIS: 1 Ta/T1 G3: 18 Ta/T1 G1/G2: 19	IL-2 (Th1) IL-10 (Th2)	Urine	Before and 4, 6, 8 h after each BCG	81 mg Connaught 6 weekly 3 weekly maintenance (36 mo)	32	Recurrence Progr	Failure to detect IL-2 during BCG related to time to recurrence ($p = 0.01$) Extended BCG cycles have high IL-2 and improved recurrence/progr-free survival
Kaempfer et al. [48]	*n* = 73 Papillary tumours: 51 CIS: 22	IL-2 mRNA IFN-γ mRNA	PBMC	Before third and fourth BCG	150 mg Pasteur 6 weekly	48	Recurrence	IL-2 mRNA induction in recurrence-free patients ($p = 0.0001$) Patients with highest IL-2 mRNA: disease-free for 30 mo ($p < 0.001$) 22/23 with relapse/persistence lacked inducibility of IL-2 mRNA 35/50 recurrence-free patients exhibited inducibility
IL-8, IL-18								
Thalmann et al. [49]	*n* = 20 NMIBC: 13 Upper tract tumours: 7	IL-8	Urine	6-h intervals after BCG	120 mg Pasteur	36.5	Recurrence Progr	Urinary IL-8 >4000 ng in 6 h after BCG in all (*n* = 10) recurrence-free patients Urinary IL-8 <4000 ng in 6 h after BCG in 9/10 recurrent patients Urinary IL-8 <4000 ng in 6 h after BCG = higher recurrence/progr risk ($p < 0.0002$)

Table 2 (*Continued*)

Study	Type of patient, No.	Type of marker	Sample type	Time of collection	BCG	Median follow-up, mo	End point	Results
Thalmann et al. [53]	$n = 28$ IL-8 measurement: 28 IL-18 measurement: 17 Ta: 21 T1: 7 Concomitant CIS: 3	IL-8 IL-18	Urine	6 h after BCG 1 (IL-8) 12 h after BCG 1 (IL-18)	120 mg Pasteur	66 (IL-8)	Recurrence	Urinary IL-8 >4000 ng = increased chance recurrence-free ($p = 0.021$) High urinary IL-18 levels = longer recurrence-free survival ($p = 0.046$)
Kumar et al. [50]	$n = 26$ BCG low (40 mg): 13 BCG standard (150 mg): 13 Low: TaG2: 4; T1G1: 4; T1G2: 4; T1G3: 1 Standard: TaG2: 5; TaG3: 1; T1G1: 3; T1G2: 4	IL-8	Urine	Before each BCG 2 and 4 h after each BCG	40 mg Danish 150 mg Danish 6 weekly	24	Recurrence Progr	Higher IL-8 levels 4 h after BCG 1 in responders ($p = 0.001$) All recurrence- and progr-free patients had IL-8 >400 pg/ml
Sagnak et al. [51]	$n = 41$ Ta: 21 T1: 20	IL-8	Urine	Before treatment after 2, 4 h at each BCG	81 mg 6 weekly	21	Recurrence	Urinary IL-8 levels after first BCG predict rec ($p = 0.047$) Urinary IL-8 levels associated with recurrence-free survival ($p = 0.006$).
Rabinowitz et al. [52]	$n = 46$ G1/G2: 25 G3/CIS: 21 TRAIL	IL-8	Urine	Before first and sixth BCG	81 mg Connaught 6 weekly	20.9	Recurrence	Pretreatment urinary IL-8 levels not associated with recurrence-free survival Increase/decrease of urinary IL-8 not related to BCG response
Ludwig et al. [56]	$n = 18$ Nonresponders: 6 Responders: 11	TRAIL	Urine	2–12 h after fifth or sixth BCG 6 Each void 24 h after BCG		12	Recurrence	Responders: higher levels of TRAIL ($p < 0.05$) Nonresponders: low TRAIL for all induction courses Responders: low TRAIL levels at BCG 1 and subsequent increase

multif = multifocal; pap TCC = papillary transitional cell carcinoma; NMIBC = non–muscle-invasive bladder cancer; BCG = bacillus Calmette-Guérin; Tis/CIS = carcinoma in situ; TAM = tumour-associated macrophage; DC = dendritic cell; FFPE = formalin-fixed paraffin-embedded; TIDC = tumour-infiltrating dendritic cell; TUR = transurethral resection; TURB = transurethral resection of bladder; PBMC = peripheral blood monocytic cell; progr = progression; TRAIL = tumour necrosis factor apoptosis-inducing ligand.

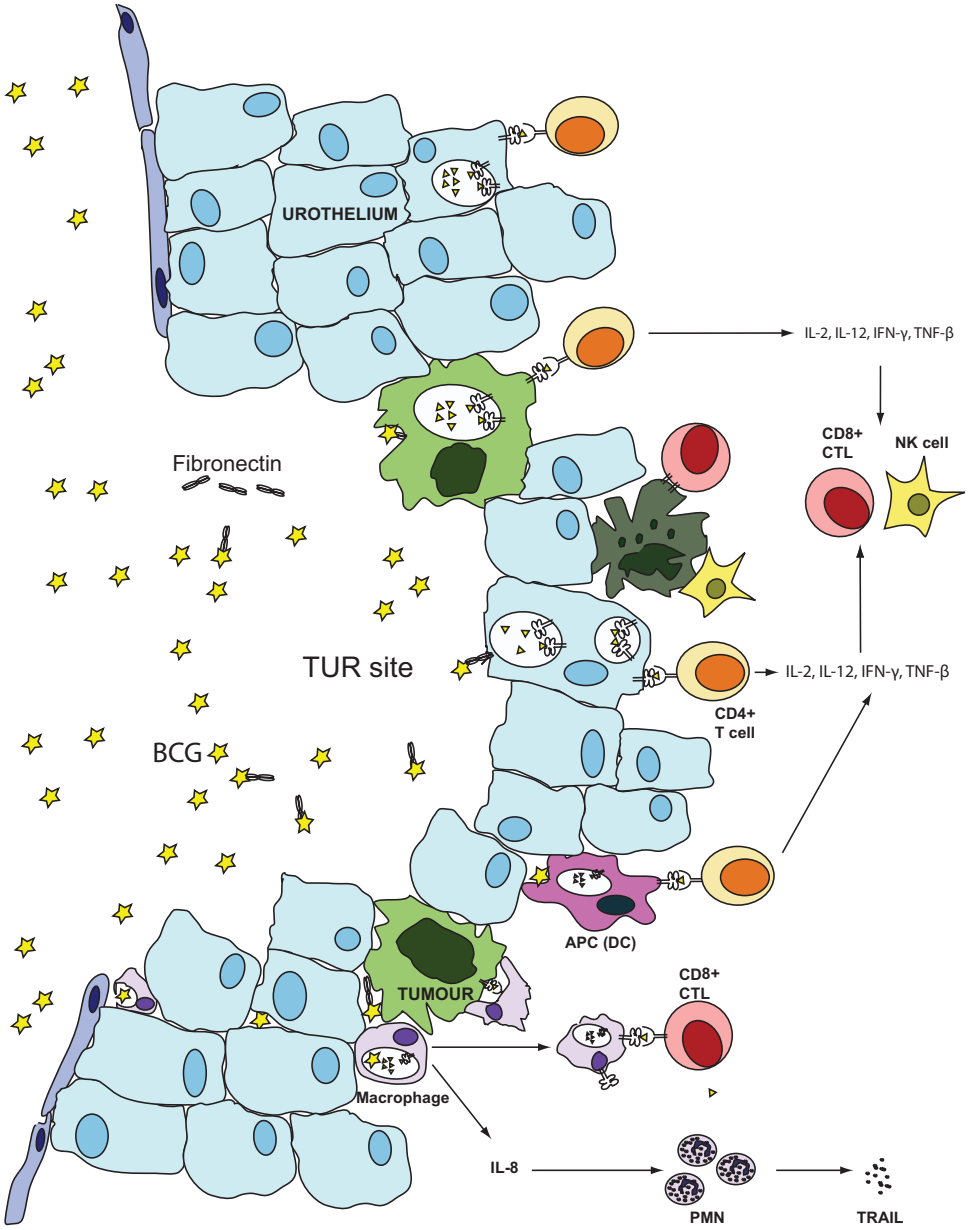

Fig. 3 – Bacillus Calmette-Guérin (BCG)–induced immune response. After internalisation of fibronectin–BCG complexes by normal urothelial and tumour cells at the transurethral resection (TUR) site, BCG antigens are presented at the cell surface, attracting $CD4^+$ T cells. Urothelial cells start a T helper-1 (Th-1) response by releasing several inflammatory cytokines (ie, interleukin [IL] 2, IL-8, IL-12, interferon [IFN] γ and tumour necrosis factor [TNF] β), which then leads to a cellular response by the recruitment of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. See text for more details.
APC = antigen-presenting cells; DC = dendritic cell; PMN = polymorphonuclear neutrophil; TRAIL = tumour necrosis factor apoptosis-inducing ligand.

studies found that high levels of urinary IL-2 after BCG administration were associated with an increased recurrence-free survival (RFS) [44–46]. Interestingly, in a more recent publication, Saint et al. showed that an extended BCG induction course first leads to urinary IL-2 overproduction, which gradually switches to a less favourable IL-10 (Th-2) profile. This suggests that some patients may not benefit from extended BCG courses, and the decision to continue extended BCG may be adapted to individual urinary cytokine levels [47]. Kaempfer et al. were the first to provide direct evidence for the concept that expression of the IL-2 gene in peripheral blood mononuclear cells is essential in BCG-induced antitumour response. They showed that 95% of BCG-NRs lacked inducibility of IL-2 mRNA, whereas 70% of the BCG-Rs (BCG responders) exhibited inducibility. They also demonstrated that IL-2 inducibility was not only significantly associated with an increased disease-free interval but that IL-2 was also an independent predictive parameter [48].

Thus measurement of IL-2 levels in blood and urine seems a promising parameter to predict BCG response. Accordingly, it would be interesting to validate these results in larger studies and to investigate the addition of recombinant IL-2 to the instillation to improve the BCG-mediated immune reaction.

3.3.3.2. Interleukin-8 and interleukin-18. IL-8 is secreted by activated macrophages, leading to the recruitment of PMN granulocytes (Fig. 3) [49]. Although high levels of urinary IL-8 measured within the first 6 h after BCG administration were associated with an increased RFS, different cut-off values were used between studies [49–51]. In contrast, Rabinowitz et al. found that urinary IL-8 levels measured before BCG instillation could not predict BCG response [52].

BCG-activated macrophages also secrete IL-18, which then activates NK cells and CTLs. Elevated levels of urinary IL-18, measured within the first 12 h after BCG administration, were significantly associated with an increased disease-free survival [53].

Thus urinary IL-8 and IL-18 concentrations measured within the first 12 h after BCG instillation could predict therapy response; however, the current use of variable cut-off values needs to be standardised.

3.3.3.3. Polymorphonuclear neutrophil granulocytes and tumour necrosis factor apoptosis-inducing ligand. High IL-8 levels lead to a massive influx of PMN granulocytes into the bladder [32]. De Boer et al. demonstrated that the content of immune cells in the urine of patients treated with BCG consists of 1–3% T cells and NK cells, 5–10% macrophages, and 75% PMN granulocytes. It seems reasonable that PMN granulocytes play an important role during BCG-induced immune response [54]. In vivo depletion of these cells from BCG-treated mice significantly impaired the influx of $CD4^+$ T cells into the bladder, thereby eliminating the effect of BCG and resulting in a decreased survival compared with nondepleted mice [33].

TRAIL is known to have apoptosis-inducing properties as well as other immunologic functions [55]. The protein can be expressed on the cell membrane or secreted as an active soluble form. Simons et al. suggested a direct antitumour effect of PMN granulocytes through the release of TRAIL [32]. They demonstrated that high levels of urinary TRAIL were present in BCG-Rs and that they effectively eradicated bladder tumour cells in vitro [56]. Interestingly, the same group showed no difference in TRAIL release from in vitro stimulated PMN granulocytes with either alive or heat-killed BCG [57]. These results are supported by a previous study from De Boer et al, who demonstrated a similar Th-1 and Th-2 cytokine pattern in mice that were first treated three times with viable BCG and subsequently received three instillations with inactivated BCG [58].

Hence TRAIL seems a promising marker to predict response and could be of importance in the current treatment strategy by reducing BCG side effects.

3.4. *Intracellular markers*

3.4.1. *TP53*

TP53 acts as a tumour suppressor protein and induces cell cycle arrest or apoptosis upon DNA damage or other cellular stress. Association between TP53 expression and BCG response was investigated in multiple immunohistochemical papers; however, none of these studies demonstrated a difference between BCG-Rs and BCG-NRs, and therefore they concluded that TP53 is not suitable as a marker to predict BCG response (Table 3) [59–62]. In contrast, other papers stated that TP53 has the potential to be used as an independent marker to distinguish between BCG-Rs and BCG-NRs in terms of time to recurrence and progression, progression to muscle-invasive disease, time to bladder cancer–associated death, and progression-free survival [19,63,64].

Thus whereas most studies suggest that high TP53 expression is a good marker to predict BCG response, some studies state the opposite. There are no obvious differences between the studies with respect to patient characteristics and cut-off values. It is therefore likely that the discrepant findings are due to statistical errors introduced by small numbers of patients and by interobserver variation in assessing P53 staining. More quantitative techniques, such as next-generation sequencing, may eventually overcome these problems.

3.4.2. *Ki-67*

Ki-67, a marker of cellular proliferation, has been found to significantly predict recurrence following BCG treatment; however, its levels were not a predictive marker for overall disease-free survival (Table 3) [64,65]. In contrast, Zlotta et al. determined Ki-67 levels as not predictive of recurrence in BCG-treated patients in their multivariate analysis [62]. Because there are no obvious differences among these three studies that might explain their discrepant findings, Ki-67 expression levels are still not suitable to predict response to BCG therapy.

3.4.3. *Retinoblastoma*

The retinoblastoma (RB) protein is a tumour suppressor involved in cell cycle control (Table 3). Esuvaranathan et al.

Table 3 – Cell-cycle markers to predict bacillus Calmette-Guérin response in non–muscle-invasive bladder cancer patients

Study	Patients	Marker	BCG	Median follow-up, mo	End point	Results
Esuvaranathan et al. [59]	*n* = 80	P53	81 mg Connaught	54	Recurrence	pRB expression not associated with outcome after BCG
	Tis: 22	pRB	27 mg Connaught		Progression	Low pRB associated with tumour recurrence ($p = 0.047$) after BCG plus IFN-γ
	Ta: 19		27 mg Connaught plus IFN-γ2b		Cancer-specific death	Low-grade tumours associated with pRB overexpression ($p = 0.019$)
	T1: 39		6 weekly plus booster			P53 expression not associated with any outcome
Lebret et al. [60]	*n* = 35 T1G3: 28 T1G3 plus CIS: 7 Analysed: 25	P53	75 mg Pasteur 6 weekly	51.3	T1 or CIS recurrence	P53 expression not associated with any outcome
Peyromaure et al. [61]	*n* = 29 T1G3: 29	P53	75 mg Pasteur 6 weekly	36.7	Recurrence Progression	P53 expression not associated with any outcome
Zlotta et al. [62]	*n* = 41	P53	120 mg Pasteur	24.6	Time to recurrence	Ki-67 high predicts BCG response ($p = 0.04$)
	TaG1: 9	Ki-67	6 weekly		Time to progression	P53 expression not associated with any outcome
	TaG2: 7	P21	Residual disease: 6 extra BCG			P21 high and Ki-67 high associated with decreased recurrence-free survival (both $p = 0.02$)
	TaG3: 2					Stage only predictor of recurrence in multivariate analysis ($p = 0.0058$)
	T1G1: 5 T1G2: 13 T1G3: 8 Tis: 3 Associated CIS: 7					
Saint et al. [63]*	*n* = 102 CIS: 2 Ta/T1G3 plus CIS: 16	P53	150 mg Pasteur 81 mg Connaught 6 weekly	40	Recurrence Progression Cancer death	Recurrence earlier positive P53 ($p = 0.01$) Cancer death higher when P53 high ($p = 0.002$) Increased risk of progression in P53 positives ($p = 0.001$)
	Ta/T1G3: 22		Pasteur no maintenance			
	Ta/T1 G1/G2: 62		Connaught plus maintenance			
Lopez-Beltran et al. [19]	*n* = 51	Cyclin D1/D3	$2–5 \times 10^{-}$ 8 CFU Tice	63.8	Disease-free survival	P27 ($p < 0.001$), cyclin D3 ($p = 0.02$), Ki-67 ($p = 0.0034$; univ)
	T1G3: 51	Ki-67	6 weekly			
		P21			Progression-free survival	P53 ($p = 0.039$), cyclin D1 ($p < 0.001$), cyclin D3 ($p = 0.001$), Ki-67 ($p = 0.016$ univ)
		P27				
		P53			Overall survival	Cyclin D3 ($p = 0.005$), Ki-67 ($p = 0.045$ univ)
Palou et al. [64]	*n* = 92	Ezrin	6 weekly	90.5	Recurrence	Ezrin independent prognostic marker of progression ($p = 0.031$)
	T1G3: 37	P53				P53 independent prognostic factor for progression to muscle-invasive disease ($p = 0.018$)
	T1G3 plus CIS: 55	Ki-67			Progression Disease-free survival	Ezrin independent prognostic marker of disease-free survival ($p = 0.035$)
Lebret et al. [65]	*n* = 35 T1G3: 35 Analysed: 25	Ki-67	75 mg Pasteur 6 weekly	57.3	Recurrence Progression	Ki-67 expression associated with clinical response ($p = 0.03$)
Cormio et al. [66]	*n* = 27 T1G3: 27	pRB	75 mg Pasteur 6 weekly Maintenance	60	Recurrence Progression Progression-free survival Disease-free survival	Progression-free survival decreased with altered pRB ($p = 0.018$) Decreased disease-free survival with altered pRB ($p = 0.037$)

Tis/CIS = carcinoma in situ; BCG = bacillus Calmette-Guérin; IFN = interferon; univ = univariate analysis.
* Restaging transurethral resection has been implemented in study.

found that low expression of RB in patients treated with BCG and IFN-α is associated with a high recurrence rate [59]. In a homogeneous population of T1G3 patients, Cormio et al. demonstrated that an altered (ie, increased or no expression) of RB was associated with decreased progression-free and disease-free survival [66]. All in all, altered RB expression could possibly serve as a predictive maker of BCG treatment outcome; however, the findings are preliminary and more studies are needed.

3.4.4. Ezrin

In 2009, Palou et al. demonstrated that low expression levels of the ezrin protein are significantly correlated with high proliferation rates in tumours of BCG-treated patients, as revealed by strong coexpression levels of Ki-67 [64]. Ezrin is involved in cell survival, adhesion, migration, and invasion. Patients with low ezrin expression levels showed a significantly poorer disease-specific survival and an increased risk of progression to muscle-invasive disease. Hence ezrin levels could identify patients who may require a more aggressive therapeutic approach than BCG treatment.

3.4.5. Combination of markers

Because carcinogenesis is a multistep process and most proteins involved are connected to each other through the various pathways, several groups investigated the expression levels of a combination of tumour suppressor proteins. Although the combination TP53/RB failed as a predictor of BCG response in a Kaplan-Meier analysis (Table 3) [59], time to recurrence was significantly increased in patients with low expression of both P21 and TP53 [62].

A single predictive marker seems insufficient due to the interconnected pathways through which proteins exert their effects, so it would be valuable to investigate the predictive value of more combinations of biomarkers in additional studies.

3.5. Gene polymorphisms

Gene polymorphisms are DNA sequence alterations within the population, affecting the predisposition to bladder cancer, response to toxins (smoking), response to treatment (BCG), and response to pathogens, thus possibly holding the key to personalised medicine. Because polymorphisms may influence gene expression, they could affect response to BCG; thus their clinical value is worth investigating (Table 4).

3.5.1. Nucleotide excision repair gene polymorphisms

Nucleotide excision repair (NER) is one of the major cellular DNA repair pathways. Variant alleles of genes involved in NER were shown to be associated with a more efficient DNA repair capacity compared with the wild-type allele [67] and thus with an unfavourable treatment outcome [68,69]. Accordingly, decreased RFS was observed in patients with variant *XPA, ERCC6, XRCC1*, *XPC*, and *ERCC2* alleles in BCG-treated patients [70–73].

Accordingly, polymorphisms in NER genes seem promising to predict RFS after BCG treatment; however, only a few studies have been validated in independent patient cohorts of different ethnicities. It would be of interest to develop a panel of high-risk alleles to predict BCG response.

3.5.2. Cytokine gene polymorphisms

Because persistent inflammation can contribute to cancer, research has been performed on genetic variations in inflammation genes and their role in the clinical outcome of BCG treatment.

IL-6 is a proinflammatory cytokine, secreted by T cells and macrophages. Studies on IL-6 polymorphisms revealed contradictory results, where the same *IL-6* SNP (−174 C/C) was either associated with an increased recurrence risk in Caucasians [74] or with a decreased recurrence risk and an increased RFS in North Indian patients [75]. *TNF-α*, a cytokine able to activate macrophages, contains gene polymorphisms associated with a decreased recurrence risk and increased RFS in BCG-treated patients [76]. The increased production of TNF-α due to the C/C polymorphism may contribute to the BCG-induced immune response, possibly extending RFS [77]. Lastly, Ahirwar et al. found that *IL-8* (−251 A/A) was significantly associated with a decreased recurrence risk and an increased RFS in BCG-treated patients [78]. IL-8 is one of the first cytokines to be released after BCG treatment and attracts PMN granulocytes, and its high expression due to the variant genotype may strengthen the immune system, resulting in improved therapy response. In the same patient cohort, they found that the *NF-κB* homozygous −94 ATTG deletion (*NF-κB* Del/Del) was associated with an adverse clinical outcome after BCG treatment. NF-κB seems to induce *IL-8* and other proinflammatory cytokines, and in this context, the Del/Del genotype could lead to lower levels of NF-κB, resulting in decreased levels of proinflammatory cytokines, possibly explaining the decreased RFS.

Several anti-inflammatory cytokines have also been investigated for genetic variants. A SNP in the peroxisome proliferator-activated receptor γ (*PPARG*) was associated with a reduced recurrence risk in nontreated patients [74]. Additionally, higher prevalence of a *TGF-ß1* variant (codon 25 G/G) was found in BCG-NRs compared with BCG-Rs [79]. Interestingly, a different SNP in *TGF-ß* (+28 T/T) was associated with a decreased recurrence risk after BCG therapy [80]. A higher production of *TGF-ß* due to this latter variant could possibly enhance apoptosis of cancer cells in addition to therapy, explaining the reduced recurrence risk.

Thus detection of cytokine gene polymorphisms seems promising, but most studies have been performed by one group in a North Indian population. The discrepant findings on *IL-6* (−174 C/C), probably resulting from genetic differences between populations, show the need for validation in different ethnicities. *IL-8* (−251 A/A) has been associated with a decreased recurrence risk, and it would be of interest to combine the polymorphism profile with the urinary *IL-8* cytokine levels to predict BCG response.

3.5.3. Other genes

NRAMP1 is the human counterpart of the murine *Bcg* gene, which controls the responsiveness to BCG treatment. The

Table 4 – Gene polymorphisms and gene signatures predicting bacillus Calmette-Guérin response in non–muscle-invasive bladder cancer patients

Study	No. of patients	No. of patients treated with BCG	BCG	Gene	Median follow-up, mo	End point	Results
Nucleotide excision repair gene polymorphisms							
Gu et al. [70]	*n* = 208 NMIBC Caucasians	Nontreated: 96 Ind: 75 Ind plus maintenance: 37	6 weekly Maintenance	*XPA* (5′ UTR) *XPC* (intron 11, poly AT) *XPC* (Ala499Val) *XPC* (Lys939Gln) *XPD* (Asp312Asn) *XPD* (Lys751Gln) *XPG* (His1104Asp) *ERCC1* (G/T at 3′ UTR) *ERCC6* (Met1097Val) *ERCC6* (Arg1230Pro)	21.7	Recurrence-free survival	Shorter recurrence-free survival after BCG treatment with *XPA* ($p = 0.078$) or *ERCC6* ($p = 0.022$)
Mittal et al. [71]	*n* = 96, NMIBC North Indian	Nontreated: 35 Ind: 54 Ind plus maintenance: 7 MMC: 16	6 weekly	*XRCC1* (Arg194Trp) *XRCC1* (Arg280His) *XRCC1* (Arg399Gln)	14	Recurrence-free survival	Fivefold increased recurrence risk after BCG in XRCC1 (399 A/A) ($p = 0.01$) XRCC1 (399 A/A) lower recurrence-free survival after BCG ($p = 0.004$)
Gangwar et al. [73]	*n* = 145 NMIBC North Indian	Nontreated: 56 Ind plus maintenance: 77	6 weekly	*XPC* (Lys939Gln) *XPC* (poly AT indel intron 9)	14	Recurrence risk Recurrence-free survival	Higher recurrence risk after BCG in *XPC* (939 C/C) (HR = 3.21; $p = 0.03$) Lower recurrence-free survival in *XPC* after BCG (939 C/C) ($p = 0.045$)
Gangawar et al. [72]	*n* = 135 NMIBC	Nontreated: 61 Ind plus maintenance: 74		*ERCC2* (Asp312Asn) *ERCC2* (Lys751Gln) *APEX1* (Asp148Glu)		Recurrence risk Recurrence-free survival	Higher recurrence risk in *ERCC2* (312 A/A) after BCG (HR = 3.07; $p = 0.016$) Shorter recurrence-free survival in *ERCC2* after BCG (312 A/A) ($p = 0.005$)
Cytokine gene polymorphisms							
Leibovici et al. [74]	*n* = 233 NMIBC Caucasians	Nontreated: 98 Ind: 85 Ind plus maintenance: 38 Cytotoxic: 12	6 weekly Maintenance SWOG protocol	*IL-6* (−174 G-C) *IL-8* (−251 T-A) *TNF-γ* (−308 G-A) *PPARG* (Pro12Ala)	20.8	Recurrence Progression	Increased recurrence risk in *IL-6* (−174 C/C) after BCG (HR = 4.31 G/C; HR = 5.47 C/C)
Basturk et al. [79]	*n* = 60 NMIBC	60	75 mg, Pasteur 6 weekly Maintenance add 6 monthly	*IL-1α* (−889 T/C) *IL-1β* (−511 T/C) (+3962 T/C) *IL-1R(a)* (1970 C/T) (11,100 T/C) *IL-2* (−330) (+166) *IL-4* (−1098 T/C) (−590 C/T) (−33 C/T) *IL-4a* (+1092 T/C) *IL-6* (−174) (+565) *IL-10* (−1082 G/A) (−819 C/T) (−592 A/C) *IL-12* (−1188 C/A)	NA	Recurrence Progression	* Higher frequencies of *TGF-β* codon 25 G/G in BCG nonresponders vs BCG responders (93% vs 64%; OR = 7.17; $p = 0.04$) Higher frequencies of *IL-4* (−1098 G/G) in BCG nonresponders vs BCG responders (17% vs 0%; OR = 18.33; $p = 0.05$) Higher frequencies of *IL-10* (−1082 G/G) in BCG nonresponders vs BCG responders (29% vs 7%; OR = 5.47; $p = 0.05$) Higher frequencies of *IL-10* (−1082 GCC/GCC) in BCG nonresponders vs BCG responders (29% vs 5%; OR = 8.4; $p = 0.025$)

Table 4 – Gene polymorphisms and gene signatures predicting bacillus Calmette-Guérin response in non–muscle-invasive bladder cancer patients

Study	No. of patients	No. of patients treated with BCG	BCG	Gene	Median follow-up, mo	End point	Results
				IFN-γ (+5644 UTR) *TGF-β* (+10 T/C) (+25 C/G) *TNF-β* (−308 A/C) (−238 A/C)			
Ahirwar et al. [75]	*n* = 113 NMIBC North Indian	Nontreated: 21, Ind: 60 Ind plus maintenance: 9 MMC: 23	6 weekly	*IL-4* intron 3 *IL-6* (−174 G/C) *TNF* (−308 G/A)	13	Recurrence	*IL6* (−174 C/C) associated with a decreased recurrence risk (HR = 0.298; $p = 0.03$) and an increased recurrence-free survival ($p = 0.021$) after BCG *IL-4* and *TNF* alleles not associated with outcomes after BCG treatment
Ahirwar et al. [76]	*n* = 147 NMIBC North Indian	Ind: 64 Ind plus maintenance: 9 Danish: 73 Strain: 1331	Danish 1331 6 weekly	*IL-1β* −511 C/T) *IL-1* RN VNTR *TGF-β* (+28 C/T) *IFN-γ* (+874 T/A)		Recurrence	Reduced recurrence risk in *TGF-β* (+28 T/T) after BCG (HR = 0.037) Increased recurrence risk in *IFN-γ* (+874 T/A) after BCG (HR = 2.8) Higher recurrence risk in IFN-γ (A/A or T/A) (HR = 2.2)
Ahirwar et al. [80]	*n* = 134 NMIBC North Indian	Nontreated: 46 Ind: 65 Ind plus maintenance: 8 MMC: 15	Danish 1331 6 weekly	*TNF-α* (−1031 T/C) (−863 C/A) (+857 C/T)	14	Recurrence	Decreased recurrence risk in *TNF-α* (−1031 C/C) after BCG (HR = 0.38; $p = 0.024$) Increased recurrence-free survival in *TNF-α* ($p = 0.024$) (−863 C/A) or (−857 C/T) not associated with outcome after BCG
Ahirwar et al. [78]	*n* = 150 NMIBC North Indian	Ind: 63 Ind plus maintenance: 8	6 weekly	*IL-8* (−251 T-A); (+678 C-T) *NF-κB* (−94 ATTG insertion/deletion)	N/A	Recurrence	Reduced recurrence risk in *IL-8* (−251 A/A) after BCG (HR = 0.12; $p < 0.001$) Increased recurrence-free survival in *IL-8* (−251 A/A) after BCG ($p < 0.001$) Increased recurrence-free survival in *NF-κB* (ins/ins) vs (del/del) after BCG ($p = 0.030$)
Other genes							
Decobert et al. [81]	*n* = 104 French Canadian	Only TUR; no recurrence: 37 Ind: 67 Controls: 109	120 mg Pasteur 6 weekly Maintenance 1–3 weekly	*NRAMP1* (GT)n *NRAMP1* (274 C/T) *NRAMP1* (469 + 14 G/C) *NRAMP1* (1465-85 G/A) *NRAMP1* (D543N G/A)	44	Recurrence	* Higher recurrence risk in (GT)n 2:3 vs 3:3 in patients with D543N G/G (HR = 2.4; $p = 0.0186$) * Higher recurrence risk in (GT)n 3:3 in patients with D543N G/A vs G/G (HR = 5.35; $p = 0.0007$)
Gene signatures							
Kim et al. [82]	*n* = 80, T1 48, training 32, validation	Ind: 80	12.5 mg Tice 6 weekly	Gene expression signature	Test set: 60 validation set: 56.2	Recurrence Progression	Gene signature independent predictor of recurrence (HR = 3.38; $p = 0.048$) and progression after BCG (HR = 10.49, $p = 0.042$) in a multivariate analysis

BCG = bacillus Calmette-Guérin; NMIBC = non–muscle-invasive bladder cancer; ind = induction; TUR = transurethral resection; HR = hazard ratio; MMC = mitomycin C; SWOG = Southwest Oncology Group; NA = not applicable.

gene regulates intracellular pathogen proliferation and macrophage inflammatory responses. Of the five polymorphisms investigated in *NRAMP1*, two variants showed an association with RFS following BCG treatment [81].

3.5.4. Gene expression

Microarray technology, enabling the evaluation of thousands of genes simultaneously, has not yet been fully used in the search for BCG response biomarkers. Until now, only one paper reported a gene signature that was able to predict BCG response and progression in primary T1 bladder tumours [82]. Although the study revealed 24 differentially expressed genes (12 for recurrence and 12 for progression) that seem to be predictive in the response to BCG immunotherapy, a limitation of the study was the absence of maintenance therapy in the studied cohort.

To gain a comprehensive insight into the complex molecular and immunologic events of BCG therapy, making more use of these high-throughput experimental methods is inevitable in the near future.

4. Conclusions

Management of high-risk bladder cancer patients remains one of the most difficult problems in urologic practice. Tumours are potentially lethal. Hence early identification of patients suited for bladder preservation with BCG treatment or radical cystectomy is essential. At this time the decision to preserve the bladder or to perform a cystectomy depends on a number of clinicopathologic parameters, but none are able to sufficiently identify patients for the appropriate therapeutic modality.

The most potent marker currently seems to be the measurement of urinary IL-2, whereby high levels of this cytokine after BCG treatment correlate with a more favourable outcome. Recombinant human IL-2 (rhIL-2) had already received approval by the US Food and Drug Administration for clinical use against malignant melanoma and renal cell cancer >10 yr ago [83,84]. Even though severe side effects during treatment might occur, they are generally manageable and reversible. Based on the promising overall response rates in the cancers just mentioned (16% and 15%, respectively), more randomised phase 3 clinical trials are currently underway to determine the efficacy of rhIL-2 in other tumours and chronic viral infections. Thus enhancing the immune response in NMIBC patients with a high-dose IL-2 regimen during or after BCG therapy seems a valuable adjuvant treatment modality. Additionally, improving current treatment strategies by reducing BCG side effects (eg, by the use of inactivated BCG) while enhancing BCG response (eg, by using improved cytokine immunotherapy [IL-12/chitosan]) could decrease recurrence and progression [58,85–88].

So far, single studies with immunohistochemical markers like tissue expression levels of DC, TAM, and ezrin seem promising, but larger studies are needed to validate the results. The relevance of cytokine gene polymorphisms needs to be investigated in other ethnicities. Thresholds for the detection of urinary IL-8, IL-18, and TRAIL levels needs to be standardised. Due to discrepant findings, expression levels of TP53 and Ki-67 seem unsuitable to predict BCG response.

Classical markers need to be investigated more thoroughly; however, we suggest using recent technological advances. These might provide us with a more comprehensive view of BCG response in terms of gene regulation. MicroRNA profiling, for example, has been successfully adopted in several types of tumours, where levels of oncogenic microRNAs not only correlated with therapy response but were also applicable as therapeutic targets [89–91]. In this regard, new biomarkers predicting BCG response might be discovered with advancing biomedical techniques.

BCG response is a multistep process, and in addition to the usual single markers, combining markers at different stages, like "pretreatment" (BCG antigen-presenting markers, gene polymorphisms, and gene signatures) and "during treatment" (urinary cytokines) could provide us with a nomogram to improve selection of patients for the appropriate treatment. In addition, large patient studies determining gene and protein expression profiles of the tumour itself, in combination with data on the tumour microenvironment (ie, the surrounding normal urothelium) and on the host's immunologic system (such as enzyme immunoassays on cytokines secreted in urine), are mandatory to get a comprehensive view of all mechanisms involved in BCG response.

Author contributions: Ellen C. Zwarthoff had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Zuiverloon, Nieuweboer.
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Critical revision of the manuscript for important intellectual content: Zuiverloon, Nieuweboer, Vékony, Kirkels, Bangma, Zwarthoff.
Statistical analysis: None.
Obtaining funding: None.
Administrative, technical, or material support: None.
Supervision: Zwarthoff.
Other (specify): None.

Financial disclosures: I certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/ affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: None.

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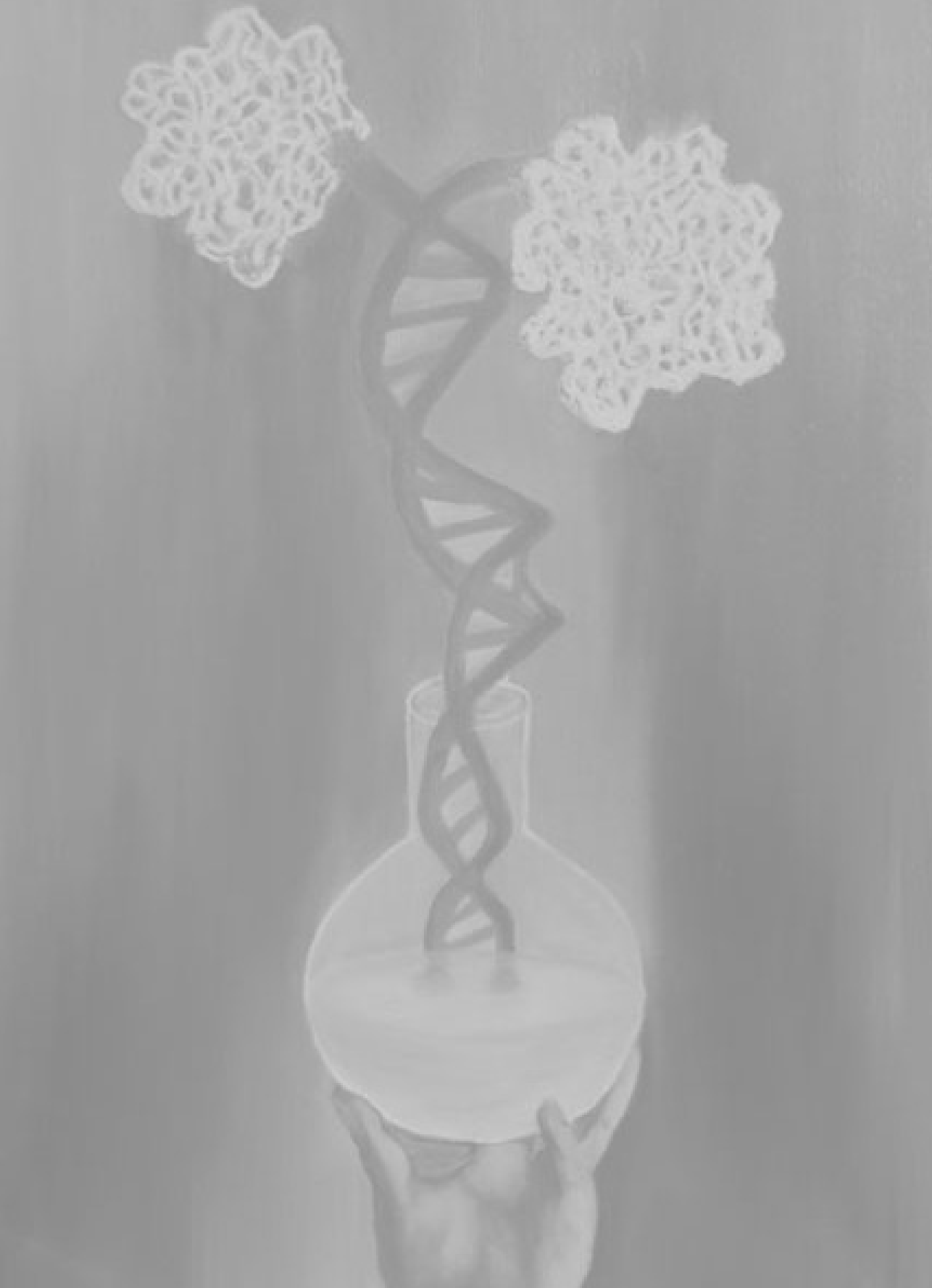

PART V
DISCUSSION AND SUMMARY

Based on:

Chapter 9
General discussion and Concluding remarks

Chapter 10
Summary and Samenvatting

General discussion and Concluding remarks

Bladder cancer is the fifth most common cancer in the world [1]. Most patients (75%) will be diagnosed with a NMI tumor and treated by TUR. These patients have a good five-year survival (80-90%), but 70% will have at least one recurrence within five years and some will even recur after 15 years of surveillance [2]. Although most NMIBC patients have recurrences of low stage and grade, progression to MI disease is observed in 10-20%. The high recurrence rate and possibility of progression to MI disease necessitate frequent monitoring of NMIBC patients. Cystoscopy and urine cytology are considered the gold standard during follow-up. Patients are stratified according to the pathologic stage and grade of the primary tumor that indicates the frequency of follow-up cystoscopies. In general, a patient will have a cystoscopic investigation of the bladder combined with urine cytology every 3-4 months. The main problems are that cystoscopy is a costly and invasive procedure causing discomfort in the patient. It has a low sensitivity for the detection of CIS and upper urinary tract tumors cannot be detected. Although new cystoscopic techniques are under investigation, up to now it is not possible to determine stage and grade of a tumor by cystoscopy. Additionally, urine cytology has a low sensitivity for the detection of low-grade tumors. Research has focused on identifying molecular markers in bladder tumors to address the above stated problems. Identification of molecular changes in bladder tumors could lead to the development of urine-based markers. The aim is to reduce the cystoscopy frequency without compromising early detection of recurrences, hereby decreasing costs and improving patient quality of life. Identification of patients at a low risk of recurrence or progression according to the molecular status of the tumor could aid in selecting patients, which can be monitored less frequently by cystoscopy.

Patients at a high risk of developing MI bladder cancer are treated with intravesical BCG instillations, but 30-50% fail to respond and 15% will have progression to MI disease. At this moment, the only strong marker to predict BCG response is refractory disease after the first or second BCG induction course or a recurrence during BCG maintenance. Markers identifying patients who will respond to BCG before or at an early phase of treatment could reduce overtreatment, costs and a delay in selection of the right treatment.

As previous research demonstrated the high incidence of *FGFR3* mutations in NMIBC patients, the first part of this thesis investigated whether *FGFR3* mutation detection could function as a diagnostic tool during surveillance **(Part 2)**. We developed a methylation detection assay to detect bladder cancer recurrences and determined whether a combination of urine-based assays could improve the current bladder cancer surveillance protocol **(Part 3)**. Lastly, we reviewed the latest findings on markers predicting BCG response in a systematic review and we proposed directions for future research studies **(Part 4)**.

Mutation detection in the *FGFR3* gene, a possible diagnostic tool?

Mutations in the *FGFR3* gene have been found in 70% of the low-grade bladder tumors and are associated with a good prognosis [3-5]. Patients with low-grade bladder tumors comprise the largest group of bladder cancer patients in the urological practice and the presence of an *FGFR3* mutation in the tumor of these patients is associated with a low risk of progression. A urine-based *FGFR3* mutation detection assay could reduce the number of cystoscopies, hereby decreasing costs and improving patient quality of life.

Chapter 2 describes a longitudinal study to determine the potential of *FGFR3* mutation analysis in urine to detect recurrences during surveillance of patients with low-grade NMIBC. Patients (n=200) were drawn from the "Cost-Effectiveness of Follow-up of patients with non-muscle invasive Bladder cancer trial" (Cefub trial) [6]. Inclusion criteria were stage Ta/T1 disease and G1/2 disease. *FGFR3* status of the tumor at inclusion was determined and urine samples were collected every three months prior to follow-up cystoscopy. We determined the sensitivity en specificity in a cross-sectional analysis, i.e. sensitivity for every follow-up urine with a concomitant cystoscopy. We found many *FGFR3* positive (mutant) urine samples without a tumor at the concomitant cystoscopy. Mutations in *FGFR3* are tumor specific and do not occur in normal tissue or urine. Therefore, we hypothesized that positive urine samples in the absence of a lesion detected by cystoscopy represent anticipatory results predicting a future recurrence and we performed a longitudinal analysis to determine the meaning of these anticipatory urine samples. Lastly, we determined the predictive value of subsequent *FGFR3* positive urine tests. Patients with an *FGFR3* mutant tumor at inclusion (n=134) were selected for analysis.

We analyzed 463 urine samples together with 45 concomitant recurrences. The sensitivity for detection of a concomitant recurrence was 26/45 (58%). Three UUT tumors were detected by the assay and missed by cystoscopy. We extended the follow-up period for at least one year after the last urine sample had been analyzed and this increased the number of recurrences to 79 (45 concomitant and 34 future recurrences). From the 105 positive urine samples, 85 (81%) were associated with a recurrence during the total follow-up period. From the other 20 positive urine samples, 8 were single positive urines within a series of negative urines and the other 12 where from patients with insufficient follow-up. It should be emphasized that from the 358 negative urine samples 317 (89%) were from patients without a recurrence and all the urine samples were concomitant with a cystoscopy. From the other 41 negative samples associated with a recurrence, most samples were within a series of positive test results or collection of only one urine sample, possibly reflecting the absence of tumor cells in the urine. A time-to-event analysis showed that a single *FGFR3* positive urine sample was associated with a three times higher risk of recurrence. Lastly, we showed that the risk of developing a recurrence increases to 90% in patients with multiple consecutive *FGFR3* positive urine samples during a period of 39 months. Thus, *FGFR3* positive urine samples are associated with a higher risk of recurrence and having multiple consecutive positive urine samples is strongly associated with development of a future recurrence. Interesting was the finding of negative test results within a series of positive tests and this led to the hypothesis that tumor cells are not always present in urine. We investigated this hypothesis in **Chapter 4**.

As most bladder cancer patients present in the sixth decade of life, only few studies investigated the molecular characteristics of bladder cancer in young patients. **Chapter 3** discusses a unique case of a 26-year-old Caucasian male, whom we encountered at our outpatient clinic and who presented with NMIBC recurring at a high frequency. Only 1-4% of the BC patients are under the age of 40 and most patients present with tumors of low stage and grade [7-9]. Some studies demonstrated that patients <20 years have tumors with a low recurrence rate, a favorable clinical outcome and few genetic alterations [10]. Despite these findings, all age groups are monitored according to the same intensive cystoscopy-based surveillance protocol. Since specifically young patients perceive a cystoscopy as burdensome, gaining more insight into the molecular changes could possibly identify a subset of patients, which can be monitored less frequently, hereby improving quality of life by reducing the number of cystoscopies. The patient presented with five multifocal recurrences (highest pTaG2) within a period of two years. He first started with BGG instillations, but switched to mitomycin-C due to complications. We analyzed multiple tumor samples for *FGFR3* mutation status, LOH on chromosome

8/9/10/11/17, *FGFR3* and TP53 expression and performed a genome-wide single nucleotide polymorphism-array (SNP). The primary tumor revealed a mutation in *FGFR3* and overexpression of the protein. LOH was detected on chromosome 9 and was confirmed by SNP-array analysis. There was no increased expression of TP53.
The molecular changes found in this case are comparable to changes found in older patients with NMI bladder tumors, possibly predicting a similar disease course. Thus, due to the risk of recurrence and progression regular controls are needed. In this case, possible risk factors that could have contributed to BC are smoking, exposure to diesel exhaust and depleted uranium. We proposed a model that explains the age related occurrence of BC and the increasing number of molecular changes at a higher age due to longer exposure to BC associated risk factors during a lifetime. We determined whether this patient was suitable for urinary *FGFR3* follow-up. All recurrences were detected with the *FGFR3* mutation analysis and the type of mutation was similar to the one found in the tumors, whereas urine cytology did not detect the recurrence in two cases. Although this concerned just one patient, our findings indicate the possibility of a urine-based follow-up in young bladder cancer patients to reduce the number of cystoscopies.
Chapter 4 describes the optimization of NMIBC recurrence detection using a urine-based *FGFR3* mutation assay. As described in Chapter 2 we found multiple cases of an *FGFR3* negative urine test within a series of positive tests. This led to the hypothesis that urine does not always contain tumor cells, possibly affecting the sensitivity of the assay. Therefore, we determined how often a urine test was positive before a TUR and whether the sensitivity of the *FGFR3* mutation assay is dependent on tumor size and the time-point of urine collection. A total of 18 patients were included with a suspect bladder lesion at cystoscopy, 8 patients had an *FGFR3* mutant tumor, 9 had no mutation and 1 patient did not have a tumor. Patients with an *FGFR3* mutant tumor were divided into tumors < 1.5cm (n=4) and > 3cm (n=4). All urines (n=440) were collected 6 days prior to surgery and analyzed by *FGFR3* mutation analysis together with the tumors. A total of 300 urine samples were analyzed in the group of patients with an *FGFR3* mutant tumor. Negative *FGFR3* urine test results were detected in 43/300 (14%) samples.
All urine samples in patients with a tumor >3cm were *FGFR3* positive, having a sensitivity of 127/127 (100%). In patients with a tumor < 1.5cm, 130/173 (75%) of the urine samples were *FGFR3* positive. We hypothesized that larger tumors shed more tumor cells into urine, leading to a higher sensitivity of the test. Additionally, it could be possible that smaller tumors shed fewer cells into the urine due to different biological characteristics or the threshold of the urine assay might be insufficient when there are fewer cells present. The sensitivity for the detection of tumors <1.5cm was improved by pooling urine samples collected over a period of 24 hours. Next, based on the assumption that morning urine is concentrated overnight, we showed that the first morning urine does not contain more tumor cells than urine collected throughout the day in both patients with a tumor <1.5cm and >3cm. This could be explained by the finding of polyuria and nocturia caused by comorbidity in these patients, such as benign prostate hyperplasia and diabetes mellitus. No mutations were detected in urine samples from patients with an *FGFR3* wild-type tumor and the one patient with no tumor, confirming the high specificity of the assay. Since most studies collect one urine sample for analysis, we find here that the sensitivity of the investigated assay is influenced by different factors, like the number of collected samples and size of the tumor. We propose a follow-up schedule for *FGFR3* mutant patients with a cystoscopy at 3, 12 and 24 months and urine analysis at 6, 9, 15, 18, 21 and 27 months for the first two years. Urine should be collected during 24 hours to achieve the highest sensitivity. A positive test result should be followed by a cystoscopy and a negative test by a urine analysis after 3 months.

We investigated the prevalence of *FGFR3* mutations in prostate cancer (PCa) in **Chapter 5.** PCa is present in 80% of men above the age of 80, but only a small proportion of these men will have clinical symptoms during their lifetime. Over the past two decades, PSA screening led to a higher detection rate of PCa cases. A large randomized controlled trial showed that PSA screening reduced cancer-specific mortality, but at the cost of overdiagnosis and overtreatment [11]. At this moment, no markers are available to identify patients at a low risk of disease progression in PCa. Mutations in *FGFR3* have mainly been associated with low risk bladder cancer and benign skin lesions. As a recent study demonstrated the presence of *FGFR3* mutations in PCa of low grade (18%), we determined the presence of *FGFR3* mutations in a group of prostate cancer patients (n=132) with primary and locally advanced disease [12]. The study by Hernandez et al. demonstrated that *FGFR3* mutations in PCa were mostly present in patients with coexistent bladder or skin tumors and we aimed to investigate this finding. In our study no mutations in *FGFR3* were detected in all investigated PCa tumors. This is in agreement with the findings from three independent earlier studies [13-15]. We discuss that the different findings could be explained by the use of needle biopsies that contain relatively low numbers of tumor cells, leading to false negative results in our study. Secondly, contamination of urothelial cells from the prostatic urethra or prostatic ducts could cause false positive results in the Spanish study and lastly, Spanish patients could differ from those in Northern Europe. *FGFR3* mutations were only detected in bladder tumors. We confirmed the presence of coexistent prostate, bladder and skin tumors and it would be of interest to investigate the signaling pathways that play a role in tumorigenesis of these tumor types.

Can bladder cancer surveillance be improved by using a combination of urine-based markers?

In the next part of the thesis, we determined whether a combination of different urine markers could possibly lead to a reduced cystoscopy frequency during follow-up. Recent studies demonstrated hypermethylation of different genes in bladder cancer and we developed a urine-based methylation detection assay that is discussed in **Chapter 6**. Hypermethylated genes have been detected in urine of bladder cancer patients and could therefore complement cystoscopic monitoring, leading to an improved surveillance protocol. Hypermethylation of genes in BC has mostly been identified in primary tumors and urine of patients with MIBC has been used to validate these hypermethylated genes in recent studies. Primary tumors are often large in size and thus shed more tumor cells than small recurrences, improving the sensitivity of tumor detection. Moreover, the fact that MI tumors are often large in size and harbor more genetic changes makes them easier to be detected. This was the first study to select genes specifically methylated in NMIBC recurrences and to validate the identified genes in urine from patients with recurrent tumors, thus simulating the real clinical setting. To this end, we designed a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay by selecting genes specifically methylated in NMI recurrent tumors (n=37) and selecting hypermethylated genes in BC from the literature. Genes methylated in urine (n=46) from non-BC patients and genes methylated in blood (n=29) from BC patients were excluded. The final assay included 23 methylation probes. A combination of four genes (*APC_a, Tert_a, Tert_b* and *EDNRB*) with the highest predictive value was selected from the MS-MLPA assay for a better clinical applicability. This smaller gene panel was selected by using a test set of urine samples collected prior to TUR (pre-TUR urine, n=68) and a control set of urines from non-BC patients (n=91). The four-gene panel reached an AUC under the ROC curve of 0.82. The gene panel was validated on an

independent data set of pre-TUR (n=49) and non-BC (n=60) urine samples. The AUC reached 0.62 in this analysis. Lastly, we determined the performance of the assay on 65 pre-TUR and 29 recurrence-free NMIBC patients. The sensitivity and specificity were 47/65 (72%) and 16/29 (55%), respectively. Interestingly, 10/11 high-risk tumors were detected and mainly pTaG1-2 tumors were missed. Other studies reported higher sensitivities, but as discussed earlier, most studies use primary tumors and urine from patients with MI tumors.
We used urine from recurrence-free BC patients as a control and believe that this reflects the true clinical practice, as patients with a history of BC have a higher basic recurrence risk than non-BC patients. We found that some tumors lack methylation of these genes and therefore we suggest patient stratification according to the methylation status of the primary tumor during surveillance.
Point mutations in *FGFR3, PIK3CA* and *RAS*, LOH and hypermethylation of specific genes have been found in bladder tumors. These molecular changes in BC can be used to develop urine-based assays for the detection of BC recurrences during follow-up, thereby possibly decreasing the number of cystoscopies in BC patients. **Chapter 7** describes the potential of single molecular markers and a combination of molecular markers previously identified in BC during follow-up of NMIBC patients. Our aim was to determine whether mutation detection of *FGFR3, PIK3CA HRAS, KRAS, NRAS,* methylation specific MLPA and MA analysis or a combination of these assays could increase the percentage of patients eligible for urine-based follow-up and to improve the sensitivity of recurrence detection during follow-up of patients with low-grade NMIBC. Results were compared to urine cytology in a retrospective longitudinal cohort that is part of the European FP7 UROMOL project. We included 136 patients with a Ta or T1 and grade 1 or 2 tumor and at least three urine samples available during follow-up. Molecular analyses were performed on the tumor at inclusion and follow-up urines (n=716). In the tumors, *FGFR3* mutations were detected in 68%, *PIK3CA* in 18% and RAS mutations in 9% of cases. A combination of the three mutation analyses increased the percentage of patients eligible for urinary follow-up to 79%. LOH was detected in 63% and methylation in 98% of the tumors. 81% of the patients had either an *FGFR3* mutation or LOH in the inclusion tumor.

Next, we determined the sensitivity of the single and combined assays for the detection of recurrences (n=552) in voided urine. Patients were stratified according to the molecular status of the inclusion tumor. *FGFR3* mutation detection reached a sensitivity of 66% and addition of *PIK3CA* and *RAS* increased this to 71%. We pointed out that only 20% of the urine samples were analyzed for *PIK3CA* and *RAS* since first MA and methylation analysis were performed. Combining *FGFR3* and MA analysis reached a sensitivity of 76%. All single and combined molecular tests had a higher sensitivity than urine cytology for the detection of recurrences.

The sensitivity of urine cytology increased from 56% to 76% combined with *FGFR3* mutation analysis without patient stratification. We argue that recurrences were missed due to the average small size of the recurrences. As described in Chapter 4, sensitivity of a urine-based assay increases with an increasing tumor size, hence we suggest that the collection of more urine samples could improve the test sensitivity. Here again, the anticipatory effect of having a positive urine sample was observed, as 85% of the *FGFR3* positive urine samples were associated with a concomitant or future recurrence. Thus, we demonstrated that the number of patients eligible for urine-based follow-up increases when combining different markers and that a combination of *FGFR3* mutation detection and urine cytology is a promising tool during follow-up.

Are there markers predicting response to intravesical BCG treatment?

Patients with high-risk bladder cancer are treated with intravesical BCG instillations. Despite the instillations, 30-50% of the patients do not respond to treatment and 15% have progression to MI disease [16-18]. Full compliance to BCG courses is affected by side effects. Additionally, a high intra- and interobserver variability among pathologists possibly leads to incorrect staging of tumors and may cause selection of the wrong treatment modality. Lastly, incomplete tumor resection is frequently reported. At this moment, BCG response is determined by refractory disease after the first or second BCG course or recurrent disease during BCG maintenance. Since high-risk BC is a lethal disease, it is advantageous to select patients as early as possible for either BCG instillations or direct cystectomy. Up to now, the only strong marker identifying patients for direct cystectomy are refractory T1 disease or CIS after BCG induction.
In **Chapter 8** we discussed important aspects concerning identification of predictive markers, reviewed the literature on clinicopathological markers, intracellular markers, inflammatory markers and gene polymorphism and provided recommendations for future research. BCG response can be measured at different phases during treatment. Early pre-treatment markers can be measured in collected blood, urine or tissue samples. Next, BCG response can be measured in blood and urine during BCG courses and finally in blood and urine after BCG treatment. We believe it is preferable to identify pre-treatment markers to avoid a delay in selecting the right treatment. Currently, urinary IL-2 measurement after BCG instillations seems promising. Moreover, high levels of urinary IL-2 have been associated with an increased recurrence-free survival and it would be interesting to investigate whether addition of IL-2 to the BCG instillations could improve treatment outcome.
There are some studies on intravesical IL-2 instillations alone that demonstrate a possible anti-tumor effect, but patient numbers are low and results have never been confirmed in larger patient cohorts. We revised other markers, but the problem with most markers remains that many studies have been performed on a single marker, using mostly small numbers of patients, different cut-off values and different time-points of sample collection between studies. Most cytokine gene polymorphism studies have been carried out in one ethnic group and need to be validated in other ethnicities. We suggest that large gene and protein expression studies could aid in the identification of suitable biomarkers. Combining the outcome of markers at different stages of treatment could lead to development of a nomogram, effectively selecting patients for the right treatment.

Future perspectives and concluding remarks

In recent years, a lot of research has focused on the identification of biomarkers in BC to improve the current gold standards, cystoscopy and cytology, during surveillance. Due to the high recurrence rate and possibility of progression, patients are monitored frequently by cystoscopy, which is an invasive and costly procedure. It is not possible to determine stage and grade of a tumor by cystoscopy and CIS is easily missed. Additionally, urine cytology lacks sensitivity. Some patients have recurrences even after 15 years of the initial diagnosis and this could result in patients being monitored during their whole lifetime. Identification of a urine-based biomarker could partly replace cystoscopies, thereby reducing the cystoscopy frequency and associated costs, improving patient's quality of life and even leading to earlier detection of recurrences. First, identification of a suitable marker for a specific group of patients is necessary.

Here, we showed that *FGFR3* mutations are a frequent finding in NMIBC patients and previous research demonstrated the association with favorable histopathological characteristics and a favorable clinical outcome. Stratification of patients with an *FGFR3* mutant tumor selects patients eligible for urine-based follow-up, thereby possibly reducing the cystoscopy frequency. *FGFR3* mutations are not present in tissue or urine of patients without bladder cancer, making the assay highly specific. The sensitivity of the assay was affected by the absence of tumor cells in urine and we demonstrated that the presence of tumors cells is dependent on tumor size and the number of collected samples. Additionally, the finding of anticipatory positive test results, i.e. positive results in the absence of a recurrence at the concomitant cystoscopy, indicates that certain patients warrant regular controls or even upper tract imaging due to the risk of developing a future recurrence. Future studies should include larger sets of patients to confirm these preliminary findings. Furthermore, more urine samples should be collected or urine should be collected during a period of 24 hours to increase test sensitivity. The next phase would be a prospective randomized controlled multicenter trial with replacement of a number of cystoscopies by urine analysis, followed by a comparison of the results to the classical cystoscopy protocol.
Since van der Aa et al. previously demonstrated that the test sensitivity increases when the urologist is aware of a positive test result, it would be interesting to include an arm into the study where test outcome is communicated to the urologist. As patients perceive the “waiting time” for a test result as the most important problem, logistics should be improved and for example the internet might be considered for communicating test results. We tested a newly developed urine preservation medium and found that urinary DNA could be preserved during seven days at room temperature and up to 14 days at four degrees (unpublished data). Since every step from urine collection to extraction and fixation of the DNA is essential for the highest test performance, this finding could improve the whole process considerably for the patients as well as researchers since there is no need for the patient to collect and deliver the urine samples directly and samples can be stored at room temperature without DNA breakdown.

In our study, all upper urinary tumors were detected by *FGFR3* analysis. Previous research found that *FGFR3* mutations occur at the same frequency in the upper urinary tract and bladder tumors harboring these mutations are also associated with a favorable clinical outcome [19, 20]. Cystoscopy is not able to detect upper urinary tract tumors and it would be very interesting to confirm these findings and to explore the possibility of a urine-based follow-up in future studies. Additionally, stratification of patients with a high risk of progression by *FGFR3* mutation status could aid in selection of the right treatment without a delay in time.
Another group that would specifically benefit from a reduced cystoscopy frequency is young patients. We proposed a model for the development of bladder tumors due to the exposure to bladder cancer associated risk factors and to the accumulation of molecular changes during a lifetime. Future multicenter studies should aim to gain more insight into the molecular pathways of BC in young patients. This could lead to identification of markers that can be used for urinary surveillance. Moreover, it would be interesting to investigate whether molecular changes found in young patients play a crucial role during early carcinogenesis. Currently, such a study to investigate the methylation status of Polycomb Group target genes, which play an important role in the differentiation of cells, is ongoing at the Erasmus Medical Center.
We showed that a combination of molecular markers increased the percentage of NMIBC patients eligible for urine-based follow-up. However, this was a single center retrospective study and validation in a prospective multicenter study is essential. Such a study is ongoing at four major European medical centers with NMIBC patients, namely “The FP7 UROMOL project”.

All urine samples and tumors of these patients will be collected and analyzed for *FGFR3* mutations and methylation status. Implementation of the discussed markers in the clinical setting is highly dependent on their cost-effectiveness. The frequent cystoscopies and TUR are costly and contribute largely to the high costs during surveillance. Identification of a marker for urine-based follow-up is the first step and making the assay cheap and easy-to-perform in even small hospitals the second step. This is specifically a challenge for MA, which is laborious and expensive, but recent data presented by van Tilborg et al. seems promising in cases where no corresponding blood is needed for comparison, considerably reducing costs and laborious efforts [21]. A virtual decision model, which was used to evaluate the cost-effectiveness of partly replacing cystoscopy by urinary *FGFR3* mutation analysis, was developed in an ongoing study at Erasmus Medical Center. Three surveillance strategies were modeled: I) standard surveillance with cystoscopy every 3 months, II) urine analysis every 3 months and cystoscopy at 3, 12 and 24 months, and III) minimal surveillance with cystoscopy every 6 months. The outcomes of recurrence, progression, survival and cumulative costs should serve as guidelines for future studies.

Selection of high-risk patients for conservative treatment or direct cystectomy still remains a challenge in the urological practice. Identification of markers predicting disease progression could aid in selecting patients for direct cystectomy. Recently, van Rhijn et al. demonstrated a favorable clinical outcome in stage T1 BC patients with an FGFR3 mutation. It would be of interest to confirm these findings in a larger patient cohort. Furthermore, identification of other markers predicting progression in high-risk tumors is important, since FGFR3 mutations are not frequently found in this group of patients. I believe it is essential to gain more insight into the underlying mechanisms of the BCG response. Understanding the initiated immune response will lead to the identification of pre-treatment markers. Specifically these markers, predicting response prior to treatment, can reduce the time delay in the current protocol for selecting the right treatment. Monitoring urinary IL-2 levels during BCG instillations seems promising, but studies on larger patient cohorts are needed to validate these results and measurement outcomes need to be standardized and comparable to outcomes of other laboratories. Since high-risk NMIBC tumors have a low incidence in the Netherlands, a future prospective study should include multiple centers.

Urine, blood and tumor tissue should be collected. I believe that analysis of normal urothelial tissue is essential. Normal tissue initiates the BCG-induced response and identification of molecular changes between BCG responders and non-responders in this tissue could give more insight into potential markers. Investigations should include for example microRNA profiling, since oncogenic microRNAs not only correlated with therapy response, but were also applicable as therapeutic targets in several tumor types. Although studies on classical markers, like expression of TP53 and Ki-67 show discrepant findings, more research is needed and results should be standardized. Bladder cancer development is a multistep process and I believe that combining different molecular markers, together with histopathological characteristics, could lead to development of a nomogram, selecting patients for the right treatment. After TUR, residual disease is detected in a considerable portion of patients and understaging of tumors is a frequent finding. For this reason, future studies should aim to evaluate and improve the current cystoscopy and TUR techniques. New techniques, like blue light cystoscopy and narrow band imaging seem promising, but the specificity of both methods is affected by prior BCG treatment. Additionally, with blue light cystoscopy patients first need to be instilled with a photosensitizer for one hour before investigation.

Pathological staging of tumors is dependent on complete resection of the tumor by the

urologist and muscle should always be present in the resected tissue. Another cause of understaging is inter- and intraobserver variability between pathologists. Molecular grading of tumors is objective and therefore preferable.
Finally, I believe that identification of BC markers could lead to the development of new treatment options, e.g. FGFR3 inhibitors. The bladder is an easily accessible organ, which can be treated locally. This is an enormous advantage compared to other tumor types. Previous promising research demonstrated the use of FGFR3 RNA inhibitors, small molecule inhibitors in BC cell lines and antibody-based targeting of FGFR3 in mice [22, 23]. Future studies should validate these results. Mutations in FGFR3 are also detected in MI tumors and indicate a good clinical outcome. Thus, FGFR3 as a therapeutic target is also advantageous in MI BC patients and perhaps could improve patient outcome.

In summary, bladder cancer is a disease that has fascinated researchers for many years. Treatment related costs are the highest compared to other tumor types and discovery of a biomarker that is able to stratify patients according to recurrence and progression risks could reduce these costs. The ultimate goal would be to develop a urine-based biomarker to reduce the cystoscopy frequency and improve patients' quality of life, but multiple factors should be considered. Urine-based surveillance does not only consider the urologist, but also the patient, the pathologist and the general health system. The test should be easy-to-perform, reproducible and easy to be interpreted. Patients should be able to understand the results and have a “save feeling”. Classical histopathological markers should be combined with molecular markers and understandable for the pathologist. Lastly, costs of an assay should be lower than a cystoscopy.

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Summary

An overview of the epidemiology, symptoms and diagnosis, pathological staging and grading, treatment options and urinary markers for surveillance of NMIBC is given in **Chapter 1**.
Chapter 2 describes a longitudinal study to determine the potential of *FGFR3* mutation analysis in urine to detect recurrences during surveillance of patients with low-grade NMIBC. Patients with an *FGFR3* mutant tumor at inclusion were selected for analysis. The cross-sectional sensitivity of the test (i.e. for every follow-up urine with a concomitant cystoscopy) was determined. The sensitivity for detection of a recurrence was 58%, including detection of three upper urinary tract tumors. We detected many positive urine samples without a tumor at the concomitant cystoscopy. Therefore, we extended the follow-up period and performed a longitudinal analysis to determine whether these positive urine samples predict a future recurrence. We found that 81% of the positive urine samples were associated with a recurrence during the total follow-up period. Additionally, we showed that 89% of the negative urine samples were not associated with a recurrence, but all these samples were concomitant with a cystoscopy. Regarding the other negative urine samples, most were in a series of positive test results, reflecting the absence of tumor cells in urine. Finally, we demonstrated that a positive urine is associated with a three-times higher risk to develop a recurrence and that having multiple consecutive positive urines during a period of 39 months increases the recurrence risk to 90%.
Most NMIBC patients present in the sixth decade of life. There is a low number of young patients presenting with bladder cancer and few studies investigated the molecular characteristics of these patients. As specifically young patients consider a cystoscopy as burdensome, we performed a case study on a 26-year-old male with NMIBC recurring at a high frequency in **Chapter 3**. We determined whether the molecular changes found in this patient were comparable to changes found in elderly patients. An *FGFR3* mutation was detected in the primary tumor and in the subsequently collected urine samples. LOH was detected on chromosome 9 and confirmed by SNP-array analysis. Expression of TP53 was not increased. The detected molecular changes were comparable to aberrations found in older patients, possibly predicting a similar disease course and warranting frequent follow-up moments. We demonstrated that all recurrences were detected by *FGFR3* urine analysis, suggesting the possibility of a urine-based follow-up by *FGFR3* urine analysis to reduce the number of cystoscopies.
We showed the presence of multiple *FGFR3* negative urine tests within a series of positive tests in **Chapter 2** and therefore, we investigated whether the sensitivity of the *FGFR3* mutation detection assay is dependent on tumor size and time-point of urine collection in **Chapter 4**. All urine samples of patients with a tumor >3cm collected prior to a TUR were *FGFR3* positive (sensitivity 100%). The sensitivity was 75% in patients with a tumor <1.5cm. We hypothesized that larger tumors shed more tumor cells into the urine, leading to a higher sensitivity of the test. We improved the sensitivity for the detection of tumors <1.5cm to 100% by virtually pooling urine samples collected over a period of 24 hours. Lastly, we showed that time-point of urine collection does not influence the sensitivity of the assay. We propose that multiple urine samples should be collected for all urine-based analyses to achieve the highest sensitivity of the test.

FGFR3 mutations in bladder cancer patients are associated with a good prognosis. In prostate cancer no markers are available to identify patients at a high risk of disease progression. A previous study demonstrated the presence of *FGFR3* mutations in prostate cancer and the association with low-grade tumors. Therefore, we determined the prevalence of *FGFR3* mutations in a large subset of primary and locally advanced prostate tumors in **Chapter 5**.

No *FGFR3* mutations were detected in the investigated tumors. This could be explained by the use of needle biopsies, which contain a relatively low number of tumor cells, leading to false negative results. False positive results in the previous study could be caused by contamination of urothelial cells or due to ethnic differences. Additionally, we investigated the prevalence of *FGFR3* mutations in prostate cancer patients with coexistent tumors in other tissues and confirmed the presence of mostly coexistent prostate, bladder and skin tumors.
Previous studies demonstrated the possibility of methylation analysis on urinary cells for the detection of bladder cancer recurrences. We developed a urine-based methylation assay specifically for the detection of NMIBC recurrences **(Chapter 6)**. To this end, we selected genes hypermethylated in recurrences of NMIBC patients and validated the results on tumor and urine samples from an independent set of NMIBC patients and urine from non-BC patients. We selected a four-gene panel (APC_a, TERT_a, TERT_b and EDNRB) that reached an AUC of 0.82 in the test cohort and 0.62 in the validation cohort. The sensitivity and specificity for the detection of recurrences in urine reached 72% and 50%, respectively. 10/11 high-risk tumors were detected using this assay. In a following study, detection of point mutations in *FGFR3, PIK3CA* and *RAS*, LOH and methylation analysis were tested in a single analysis or in a combination of markers and all were compared to urine cytology **(Chapter 7)**. *FGFR3* point mutations were present in 68% of the patients, *PIK3CA* in 18% and RAS mutations in 9% of cases and a combination of these markers increased the percentage of patients eligible for urinary follow-up to 79%. LOH was detected in 63% and methylation in 98% of the tumors. Next, we determined the sensitivity of the assays after stratification of patients according to molecular status of the primary tumor. *FGFR3* mutation detection reached a sensitivity of 66% and addition of *PIK3CA* and *RAS* increased this to 71%. A combination of *FGFR3* and LOH analysis reached a sensitivity of 76%. All single and combined tests had a higher sensitivity than urine cytology. Combining urine cytology with *FGFR3* mutation analysis increased the sensitivity from 56% to 76%.

Currently, one of the most difficult decisions in high-risk NMIBC patients is whether a patient should be treated with BCG intravesical instillations or should directly receive a radical cystectomy. There was no clear overview on the available biomarkers predicting BCG response. Therefore, we performed a systematic review on biomarkers predicting BCG response in **Chapter 8**.
We propose that markers are divided into early pre-BCG, during-BCG and post-BCG treatment markers. To prevent any time-delay in choosing the right treatment, it is preferable to identify pre-treatment markers predicting BCG response. Immunohistochemical analyses of intracellular markers like TP53, Ki-67 and Rb showed discrepant findings due to small patient samples. Multiple studies demonstrated the usefulness of cytokine gene polymorphisms analyses, but as most studies were performed in an Indian population, studies need to be performed in other ethnicities. At that time, only one gene expression microarray study was done and the use of these high-throughput methods could preselect genes involved in the BCG response that can be investigated with functional studies. At this moment, the most potent biomarker seems measurement of urinary IL-2, as high levels are associated with an increased recurrence-free survival and findings were confirmed in other studies. Other suggestions included the addition of IL-2 to BCG instillations.

Samenvatting

In **Hoofdstuk 1** wordt een kort overzicht gegeven van de epidemiologie, symptomen, diagnose, pathologische stadiëring en gradering, behandelopties en urine markers voor de follow-up van niet-spierinvasief blaaskanker (NSBK). In **Hoofdstuk 2** wordt in een longitudinale analyse de mogelijkheid van *FGFR3* mutatie analyse op urine voor de detectie van blaaskanker recidieven bij patiënten met laaggradig NSBK besproken. Patiënten met een *FGFR3* mutatie in de inclusie tumor werden geselecteerd voor de analyse. De crossectionele sensitiviteit van de test (bij iedere follow-up urine met gelijktijdig een cystoscopie) werd bepaald. De sensitiviteit voor de detectie van een recidief in de urine was 58%, waarbij tevens drie tumoren van de hogere urinewegen werden gedetecteerd. Omdat er multipele positieve urine monsters werden waargenomen in de afwezigheid van een recidief tumor bij de cystoscopie, hebben we de follow-up periode uitgebreid en een longitudinale analyse verricht om te bepalen of de positieve urines geassocieerd waren met een toekomstig recidief. 81% van de positieve urines was geassocieerd met een recidief tumor tijdens de gehele follow-up periode. 89% van de negatieve urines was niet geassocieerd met een recidief, maar al deze urines waren tegelijk met een cystoscopie afgenomen. Het grootste deel van de overige negatieve urines was temidden van een serie van positieve urines, wat zou kunnen duiden op de afwezigheid van tumorcellen in de afgenomen urine. Tot slot laten wij zien, dat het hebben van een *FGFR3* positieve urine is geassocieerd met een driemaal hogere kans op het ontwikkelen van een recidief tumor en dat de kans op een recidief oploopt tot 90% bij het hebben van multipele positieve urines gedurende een periode van 39 maanden.

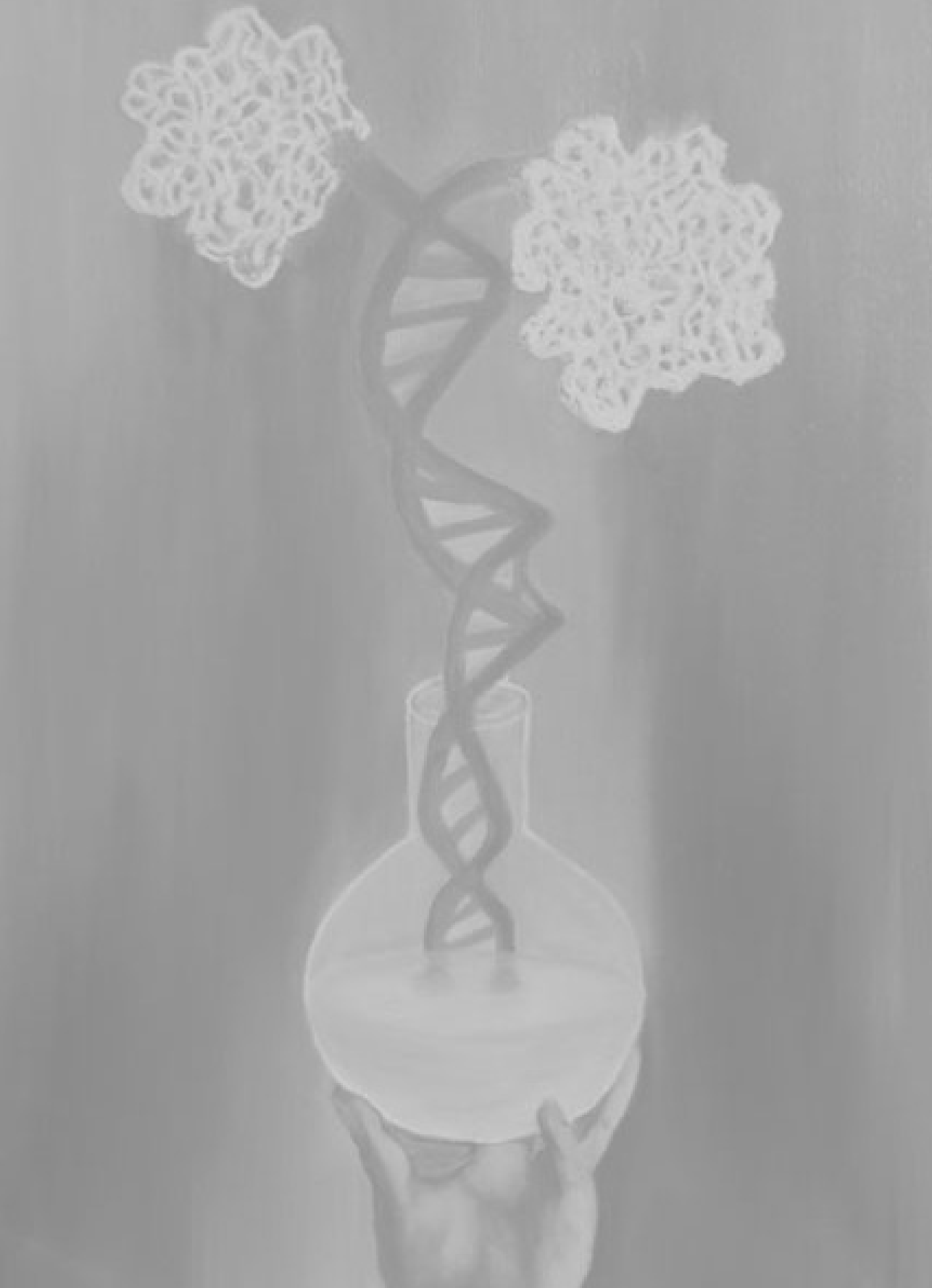
De meeste NSBK patiënten presenteren zich rond het zestigste levensjaar. Er is een kleine groep jonge patiënten met blaaskanker en er zijn slechts enkele studies die de moleculaire achtergrond van blaastumoren in jongeren hebben onderzocht. Omdat specifiek jonge patiënten de cystoscopie als storend ervaren, hebben wij een case studie uitgevoerd in een 26-jaar oude man met NSBK, die met een hoge frequentie recidiveerde **(Hoofdstuk 3)**. We hebben bepaald of de moleculaire veranderingen in deze jonge patiënt overeenkomen met de moleculaire veranderingen in oudere patiënten. Een mutatie in *FGFR3* werd gevonden in de primaire tumor en in de gedurende de follow-up verzamelde urines. LOH van chromosoom 9 werd gedetecteerd en bevestigd met SNP-analyse. TP53 expressie was niet verhoogd. De in deze studie gevonden moleculaire afwijkingen komen overeen met de afwijkingen die gevonden worden in oudere patiënten. Deze bevinding zou kunnen betekenen, dat deze patiënt een vergelijkbaar beloop van de ziekte kan hebben en frequent nagekeken moet worden. We hebben laten zien, dat alle recidieven in de urine werden gedetecteerd met de *FGFR3* mutatie analyse en dat deze techniek mogelijkheden zou kunnen bieden voor een follow-up schema met urine diagnostiek.

In **Hoofdstuk 2** demonstreren we de aanwezigheid van meerdere *FGFR3* negatieve urine testen in een serie van positieve urines. In **Hoofdstuk 4** onderzoeken we of de sensitiviteit van de urine test beïnvloedt wordt door het formaat van de tumor in de blaas of door het tijdstip van urine afname voor analyse. Alle pre-TUR urine samples afgenomen bij patiënten met een tumor >3cm waren positief voor *FGFR3* (sensitiviteit 100%). De sensitiviteit voor de detectie van een recidief was 75% in patiënten met een tumor van <1.5cm. Een mogelijke verklaring is dat grote tumoren meer tumorcellen verliezen in de urineblaas waardoor de sensitiviteit van de test toeneemt. Door virtueel urine samples, die over een periode van 24 uur verzameld waren samen te voegen, konden we de sensitiviteit verhogen tot 100%.

Tot slot tonen we aan, dat het tijdstip van urine afname geen invloed heeft op de sensitiviteit van de test. Ons voorstel is, dat bij iedere urine test meerdere samples worden afgenomen om de hoogste sensitiviteit te bereiken.
FGFR3 mutaties in blaaskanker patiënten zijn geassocieerd met een goede prognose. Op dit moment zijn er geen markers beschikbaar die hoog-risico patiënten kunnen identificeren. Een voorgaande studie toonde de aanwezigheid van *FGFR3* mutaties in prostaatkanker patiënten en de associatie met laaggradige tumoren aan. Deze bevinden wilden wij onderzoeken in een grote groep patiënten met prostaatkanker in **Hoofdstuk 5**. Er werden geen mutaties gevonden in de onderzochte tumoren. Deze negatieve bevinding zou een gevolg kunnen zijn van het gebruik van naaldbiopsie preparaten in onze studie, waarbij over het algemeen weinig tumorcellen worden verkregen. Vals positieven in de voorgaande studie zouden het gevolg kunnen zijn van contaminatie van urotheelcellen in het prostaatweefsel of etnische verschillen. Aanvullend hebben we de prevalentie van *FGFR3* mutaties bepaald in prostaatkanker patiënten met co-existente tumoren in ander weefsel. Overeenkomend met de voorgaande studie vonden wij, in het geval van meerdere tumoren in één patiënt, met name een combinatie van prostaat, blaas en huidtumoren.
In voorgaande studies werd aangetoond, dat methylerings analyse van de urine voor de detectie van blaaskanker recidieven een mogelijkheid is. Wij ontwikkelden een methylerings assay op urine materiaal, specifiek voor de detectie van NSBK recidieven **(Hoofdstuk 6)**. Hiervoor selecteerden we genen die gemethyleerd waren in recidieven van NSBK patiënten en we valideerden deze resultaten op tumor materiaal en urine van een onafhankelijke groep NSBK patiënten en urine van patiënten zonder blaaskanker. Uiteindelijk werden vier genen geselecteerd (APC_a, TERT_a, TERT_b and EDNRB), die een AUC van 0.82 hadden in het test cohort en 0.62 bij de validatie. De sensitiviteit en specificiteit voor de detectie van recidieven in de urine waren respectievelijk 72% en 50%.10/11 hoog-risico tumoren waren gedetecteerd met deze test. In de volgende studie werden detectie van puntmutaties in *FGFR3*, PIK3CA en RAS, LOH en methylerings analyse uitgevoerd als enkele test of als een combinatie, waarbij de uitkomsten werden vergeleken met urine cytologie **(Hoofdstuk 7)**. Mutaties in *FGFR3* werden aangetoond in 68% van de patiënten, PIK3CA in 18% en RAS mutaties in 9% en door de combinatie van deze drie markers liep het percentage patiënten dat geschikt was voor urine follow-up op tot 79%. LOH werd gedetecteerd in 63% van de patiënten en methylering in 98%. Vervolgens werd de sensitiviteit van de verschillende testen bepaald na patiëntstratificatie op basis van de moleculaire status van de primaire tumor. De sensitiviteit van de *FGFR3* mutatie analyse was 66% en bij aanvulling met PIK3CA en RAS mutatie analyse nam dit toe tot 71%. *FGFR3* mutatie detectie gecombineerd met LOH analyse leverde een sensitiviteit op van 76%. Alle aparte testen en combinaties van testen hadden een hogere sensitiviteit dan urine cytologie. Urine cytologie gecombineerd met *FGFR3* mutatie analyse verbeterde de sensitiviteit van 56% naar 76%.

Bij hoog-risico NSBK patiënten blijft het een zeer moeilijke beslissing of zij direct een radicale cystectomie of eerst intravesicale spoelingen met BCG moeten krijgen. Er was over de verschillende biomarkers, die BCG respons zouden kunnen voorspellen, nog geen duidelijk overzicht beschikbaar in de recente literatuur. Om die reden beschrijven wij in **Hoofdstuk 8** een systematische review over biomarkers, die de BCG respons zouden kunnen voorspellen. Wij stellen voor, dat markers worden onderverdeeld in vroege pre-BCG, tijdens-BCG en post-BCG markers. Het heeft de voorkeur vroege pre-BCG markers te identificeren en hiermee vertraging in de keuze voor de juiste behandeling te voorkomen.

Studies over immuunhistochemische markers zoals TP53, Ki-67 en Rb laten verschillende uitkomsten zien, mogelijk als gevolg van kleine patiënt aantallen. Meerdere studies demonstreren, dat analyse van gen polymorfismen in cytokine genen waardevol is, maar dat de uitvoering in enkel de Indiase populaties een beperking blijft en dat deze bevindingen eerst gevalideerd moeten worden in andere etnische groepen. Ten tijde van het schrijven van deze dissertatie was er één gen expressie microarrary studie uitgevoerd en het uitbreiden van dit type onderzoek zou genen kunnen selecteren, die betrokken zijn bij de BCG respons. Deze genen zouden aansluitend in functionele studies onderzocht kunnen worden. De meest potente biomarker is op dit moment de meting van urine IL-2 concentraties. Hoge concentraties waren geassocieerd met een verbeterde recidief-vrije overleving en gevonden in meerdere studies. Aanvullend zou het interessant zijn om de effecten van BCG spoelingen, gecombineerd met IL-2, te bepalen.



PART VI

Appendices

Abbreviations

List of publications

Acknowledgement / Dankwoord

Curriculum vitae

PhD Portfolio

Abbreviations

BC	Bladder cancer
CIS	Carcinoma in situ
PDD	Photodynamic diagnosis
NBI	Narrow band imaging
BCG	Bacillus- Calmette Guerin
CT	Computed tomography
WHO	World health organization
PUNLMP	Papillary lesion of low malignant potential
NMI	Non-muscle invasive
MI	Muscle-invasive
TUR	Trans-urethral resection
EAU	European Association of Urology
FDA	United States Food and Drug administration
FISH	Fluorescence in situ hybridization
FGFR	Fibroblast growth factor receptor
SADDAN	Severe achondroplasia with developmental delay and acanthosis nigricans
MM	Multiple myeloma
MA	Microsatellite analysis
LOH	Loss of heterozygosity
DNMT	DNA-methyltransferases
SNP	Single nucleotide polymorphism
PCa	Prostate cancer
MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification
APC	Adenomatosis polyposis coli
TERT	Telomerase reverse transcriptase
EDNRB	Endothelin receptor type B

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1. Beukers W, Hercegovac A, Vermeij M, Kandimalla R, Blok AC, van der Aa MM, Zwarthoff EC, Zuiverloon TC. Hypermethylation of the Polycomb Group Target Gene PCDH7 in Bladder Tumors from Patients of All Ages. J Urol. 2013 Jan 28

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Dankwoord

Dit was het dan, here we go!

In 2006 begon het allemaal in het lab van Ellen Zwarthoff. Een geweldige tijd waarin ik veel heb gedaan en geleerd. Nu aan mij de taak om iedereen te bedanken. Voor dit dankwoord begon ik met een lijst van mensen die ik graag wilde bedanken voor de steun gedurende deze periode. Echter werd deze lijst zo lang dat het er simpelweg op neerkomt dat ik het in deze periode enorm naar mijn zin heb gehad met iedereen en steun heb gehad van bijna alle mensen om mij heen. Toch wil ik graag nog even de spotlight richten op een paar mensen die dit zeker verdiend hebben. And the beat goes on!

Ellen, het begon bij jou in de kamer dat we lekker filosofeerden over het ontstaan van blaaskanker. Voor mijn gevoel klikte het direct en ik zal nooit vergeten dat je mij al heel snel na de sollicitatie belde terwijl ik met mijn moeder aan het varen was in België. Ik was aangenomen! Van blijdschap viel ik bijna uit de boot in het water. In mijn leven zijn er enkele mensen waar ik enorm tegen opkijk en die ik waardeer om wie ze zijn als persoon. Ellen, jij bent hier 100% één van. Je hebt mij veel geleerd en je was er altijd voor me. We konden kletsen over wijntjes en restaurants, filosoferen over ons onderzoek en natuurlijk lekker klagen over die vervelende artsen…..! Je bent kritisch, gezellig en staat altijd voor mij klaar. Ik hoop dat wij nog lang samen zullen werken.

Professor Bangma, bedankt voor de ondersteuning van mijn promotietraject en het meedenken tijdens onze maandelijkse woensdagmiddag blaasmeetings in het lab. U kijkt altijd kritisch naar de probleemstelling en komt steeds weer met leuke ideeën. Ondanks alle drukte kon ik altijd bij u terecht met vragen! Dank hiervoor.

Ewout, Arno en Guido, bedankt voor de inhoudelijke beoordeling van mijn proefschrift. Ewout ook wij hebben samen met Hester veel gefilosofeerd over hoe we urine diagnostiek in combinatie met de cystoscopie inzichtelijker kunnen maken en we gaan gewoon door. Dank Ewout en Hester. Arno, dank voor het reviewen van de coupes van Cheno en mij. Wat eerst begon als een kleine studie werd toch “iets” groter, maar je was altijd bereid om ons te helpen ondanks alle drukte bij de pathologie.

Torben and Maggie, thanks for being a member of my PhD committee and taking the time to come to the Netherlands.

Monique ook jij bedankt voor je bereidheid zitting te nemen in de grote commissie en je aanwezigheid bij de inhoudelijke verdediging van dit proefschrift.

Kamergenoten Magda Maestro, Hedy aka Olivier en Martinus, dank voor alle gezelligheid en ondersteuning. Het was altijd feest bij ons op de kamer! Magda Maestro, je hebt mij zoveel geleerd. Jouw rust en geduld om een ander iets te leren is bewonderenswaardig. Nu weet ik tot in details hoe de scanner op de afdeling, Adobe, Endnote, Pubmed, Powerpoint, Word en niet te vergeten Illustrator werken. “Our first illustrator” is in ieder geval vereeuwigd in European Urology! Hedy, waar zal ik beginnen? Borreltjes, biertjes, the source, flirtation, Maastricht, Grote markt Den Haag, Stockholm, de Paap, Amsterdam, Ypenburg, ons gezinnetje met onze Fristi drinkende dochter, en nog veel meer. Gezien het aantal woorden s.t.o.p. Ik denk dat je de strekking wel begrijpt. Natuurlijk ook dank voor het feit dat je mijn paranimf wilt zijn.

Martinus, ook jij hebt een bijzondere plek in mijn gedachten. Ik zal je nooit vergeten! Thanks for being you! Kamergenoten, ik heb een top tijd met jullie gehad!

Kirstin aka KvK, dank voor de ondersteuning tijdens het onderzoek. We hebben samen enorm veel werk verzet en veel goede discussies gehad. Je was altijd bereid om door te werken en voor de 100000ste keer weer alles na te kijken, omdat ik weereens vergeten was iets mee te tellen. Je was er ook altijd voor de studenten, die enorm veel van je hebben geleerd. Ook jij bedankt voor de gezelligheid en ondersteuning!

En dan niet te vergeten natuurlijk my students. Klein Suriname begon allemaal met Willie en Stephen. Willie, je bent een topmeid met veel potentie. We hebben samen gefeest en leuke research opgezet en ik hoop dat je de juiste keuzes maakt zodat we hiermee door kunnen gaan!!! Stephen, jij bent mijn eerste en dus altijd heel speciaal gebleven ;-). Ook wij hebben veel meegemaakt en ondanks alles waar je doorheen ging was je altijd bereid om anderen te helpen. Ook nog dank voor de ondersteuning bij de Suriname benefiet! Keep up the good spirit. Klein Suriname groeide en daar was Cheno. Cheno, dank voor je inzet bij de verschillende projecten. We hebben veel discussies gehad, maar je bent en blijft voor mij altijd een speciaal persoon. And the beat goes on. Annemiek kwam ook bij de club. De student die mij instrueerde. Tahlita, heb je het al af? Nog steeds niet! Annemiek, dank voor je inzet en je doorzettingsvermogen. We hebben er iets moois van gemaakt. Ga zo door! Jeannine, het was een korte periode waarin we ook veel hebben meegemaakt. Dank voor al je inzet. Gangsterboys Aleksander en Serdar, dank voor alle inzet en gezelligheid. Jullie hebben hard gewerkt voor het onderzoek en de urines, zelfs tot in de vroege uurtjes…. Jie-Fen en Hrvoje, thanks voor het verzamelen van de urines en netjes bijhouden van de database. Ook wij hebben met de groep een gezellige tijd gehad.
Keep up the good work! Klein Suriname was erg gezellig. Dank allemaal.

Marcel, enorm bedankt voor het maken van onze coupes. Het was niet altijd even geordend, maar jij wist orde te scheppen in onze chaos. Ook van jou heb ik veel geleerd. Dank hiervoor.

Lucie, Irene, Angela en Raju, dank voor de nodige ondersteuning. We hebben samen een gezellige tijd gehad in Be-304.

Thanks to all UROMOL members for your support and input.

Alle co-auteurs, bedankt voor jullie bijdrage aan mijn publicaties.

Natuurlijk niet te vergeten iedereen van de afdeling urologie en het OK complex, dank voor de inzet en het verzamelen van weefsel en urine. Martijn, je was altijd bereid om mee te denken hoe we nog meer urine konden verzamelen op de polikliniek.
Joost, ook jij hebt ons ondersteund bij het verzamelen van materiaal en het meedenken voor vervolgonderzoeken. Dank hiervoor.
Dr. Kirkels, dank voor het meedenken bij onze blaasmeetings. Alle personeel op de afdeling en polikliniek, tot op de dag van vandaag verzamelen jullie voor ons urine. Dit wordt zeer gewaardeerd!

Alle patiënten die bereid waren mee te doen aan ons onderzoek, hartelijk dank hiervoor. Zonder jullie kunnen wij dit onderzoek niet voortzetten.

Iedereen in het Haga Ziekenhuis bedankt voor een leuk en mooi jaar waarin ik veel heb geleerd. Special thanks to Hossein Roshani, omdat je er altijd voor me was. Ik vond het een zeer prettige samenwerking en voor mij ben ook jij een persoon waar ik veel respect voor heb. Madelon, Mareille, Bin, Sarah en Astrid, het was super gezellig.

Alle collega's in het Sint Franciscus gasthuis bedankt voor een leerzame periode. Special thanks to Rajaa. Zonder jou was het toch anders. Je hebt mij leren opereren en had geduld ondanks het feit dat je gejinxt bent. Deniz, we did it en ookal kon het niet voorin, staat het toch nog hier: what goes around comes around, karma ;-). An Jai en Sandra, members van de SFG BTCHS club. Het was super gezellg met jullie. Dank voor de ondersteuning. En Sandra als je auto het weereens niet doet kan je altijd meerijden. Dr. Strijdhorst en dr. van de Ham, dank voor de rust, kennis en operatiekunst die jullie mij hebben geleerd. Respect.

Huidige urologie collega's, Lizette, Kim, Marloes, Peter-Paul, Dorien, Tineke, Marit, Pim, en Diederick thanks voor de ondersteuning en we gaan vast nog veel meer gezellige tijden tegemoed. Fred, Joop, Katja en Jeroen, bij jullie begon het allemaal eindelijk, dank voor voor de ondersteuning die nodig was!
Paul, Willem, Gert, Marij en Bertil ik hoop nog veel van jullie te kunnen leren. Iedereen dank!

Tjerk en Amiroz, mijn levensvrienden, santé! Met jullie kan ik alles bespreken en jullie zijn er altijd voor mij. Bedankt voor alle steun, leuke reisjes, feestjes, proeverijen en get togethers.
Dat er nog veel mag volgen!

Ruby Swarma, we go back en je was er altijd voor me, meer hoef ik niet te zeggen.
Thanks hiervoor!

Ja9 ook jij hebt mij altijd gesteund in leuke en moeilijke tijden. Je bent er altijd voor me no matter what. Thanks hiervoor.

Lisa ook we go back en we hebben veel meegemaakt. Dank dat je bent wie je bent en mij onvoorwaardelijk ondersteund.

Marlies, we don't just go back, we go wayyyyy back. Dank voor je ondersteunende gesprekken op alle vlakken. We konden de zaken altijd weer samen goed relativeren.

Olaf aka Ollie, thanks dat je bent wie je bent. Je bent er altijd voor me en we hebben goede gesprekken gehad. Stay in the family!

Karin en Erlijn, dank voor alle support en leuke tijden!

Katrien and Steve-O, thanks for your support in Chicago. I had a wonderfull year with you guys.

Lindy, thanks voor de gesprekken in de mensa. Ik heb er altijd van genoten!

Lieve familie, oom Hubert, (tante) Sandra, Shiraleetje, Kasandra, Timothy, Denise, oom Andre, oom Jose, oom Tonio, Tante Martha, Joyce, Sha-ira, Shari-nischa, tante Jolly, Ome Perry, Tante Sillie, Adolfus, Heidi en Brady dank voor alle support!

Yvette, bedankt voor alle steun, goede gesprekken en leuke brunches!

Oma Poetie!!!!!! Bedankt dat je altijd voor mij bidt en aan me denkt. Je bent een top oma en ik vind het superleuk om tijd met je door te brengen. Sangriaaaa, what happens in Lissabon stays in Lissabon.

Robert, bedankt voor je ondersteuning. Ookal hebben we soms discussies weet ik dat je er altijd voor mij bent no matter what en geniet ik van onze uitjes. You my bro forever! Leonidas ook jou wil ik niet vergeten en bedanken voor alle gezelligheid en gesprekken.

Moeder en papa, bedankt dat jullie er altijd voor mij zijn. Ik weet dat het onvoorwaardelijk is. Jullie support en stimulans heeft mij gemaakt tot wie ik ben. Moeder, ookal kwam ik weer voor de 1000ste keer met een of ander ingewikkeld medisch verhaal luisterde je en kwam je met een goed advies, dat waardeer ik enorm. Jij bent mijn voorbeeld. Papa, ik moet kritisch kijken naar mijn werk, mijn houding, mijn instelling en er altijd zijn voor mijn patiënten. Jij stimuleerde mij zoals niemand anders dat kan. Je wist de medische situatie altijd te vertalen naar een situatie in de accountantspraktijk, mooi! Jij bent mijn voorbeeld. Jullie zijn mijn voorbeeld.
Bedankt voor alles in mijn leven en dit proefschrift draag ik aan jullie op!

Lieve Megan Elisabeth, bedankt voor alle steun en support. In al mijn gekte met c1000 tassen was je er onvoorwaardelijk voor me en heb je me altijd gesteund tijdens het schrijven van mijn proefschrift. Ik heb veel van je geleerd, hoop dat ik nog veel meer met je meemaak en dat we samen nog ouder worden. Zoals jij zou zeggen Omnia causa fiunt. Dus ook dit.

Tahlita

What would life be without urine?

Curriculum vitae

Personal information	o Surname : Zuiverloon o Names : Tahlita Carmelita Margarita o Date of birth : 18-05-1979, Haarlem o Nationality : Dutch o Gender : female
Education	o **2004-2006: Medical doctor degree, Leiden University** - 16 weeks final- internship surgery, Medisch Centrum Haaglanden: Westeinde - 8 weeks internship gynaecology, Paramaribo, Suriname o **2000-2004: Master degree Medicine, Leiden University** - Electives: Medical sociology and Anthropology, Problems in developing countries o **1999-2000: Bachelor degree Medicine, Leiden University** o **1998-2003: Master degree Biomedical Sciences, Leiden University** - Laboratory animal science certificate - Radiation course level 5D certificate o **1997-1998: Bachelor degree Biomedical Sciences, Leiden University** o **1992-1997: Degree Gymnasium, Katholieke Scholengemeenschap Hoofddorp**
Experience	o **2013-now: Urology resident (AIOS), Erasmus MC Rotterdam** o **2011-2012: Surgery resident (AIOS), Sint Franciscus Gasthuis Rotterdam** o **2010: Urology resident (ANIOS), HagaZiekenhuis The Hague** o **2008-2009: Urology resident (ANIOS), Erasmus MC Rotterdam** o **2006-now: PhD degree, department of Pathology, Erasmus MC Rotterdam** - Identification of molecular markers for the early detection of bladder cancer recurrences in voided urine. - Courses: Basic and translational oncology, Molecular diagnostics, Molecular imaging, NvvO Oncology course, NIHES course: Medical statistics, Biomedical English writing, Imaging for doctors, Survival analysis for doctors. o **2006: MD-volunteer, Mzuzu Central Hospital, Malawi Africa** - resident urology/surgery for 3 months o **2003-2004: Dexa botscan analyst, Bioimaging technologies Leiden** o **2002-2003: Nurse level C, Thuiszorg Rijn en Vliet, Leiden** o **2002: Nursing and guidance of HIV-positive children (0-2yrs), Bowie House, Paarl, South- Africa** - Internship 4 months - Provide education on HIV and tuberculosis

Research	o **2001-2002: The role of Notch in tumor escape mechanisms, Loyola University Cardinal Bernardin Cancer Center, Chicago, USA** - Internship 10 months, Department of Immunology, Prof. W.M. Kast o **2002: The role of small GTP-ases in T-lymphocyte development** - Literature thesis, Department of Immunology, Prof. W.M. Kast o **2001: Mutations in Mannan Binding Lectin (MBL) and the functional activity of MBL in the complement system.** - Internship 4 months, Department of nephrology, Prof. M.R. Daha o **2000: The role of cJun in transcription regulation, Leiden University** - Extra Internship 6 months department of molecular biology, Dr. Ir. H. van Dam
Conferences	o **2013 European Association of Urology, Annual meeting Milan:** Urinary biomarkers in the follow-up of low grade non-muscle invasive bladder cancer patients: Update on the FP7 UROMOL project. Poster presentation o **2011 European Association of Urology, Annual meeting Austria:** Optimalisation of detection of recurrent bladder cancer using a urine-based mutation assay. Presentation o **2011 European Association of Urology, Annual meeting Austria:** Urinary biomarkers for the detection of non-muscle invasive bladder cancer recurrences: The European UROMOL project. Presentation o **2011 European Association of Urology, Annual meeting Austria:** A urine-based methylation assay for the detection of recurrences during follow-up of non-muscle invasive bladder cancer patients. Presentation o **2010 Dutch Urology society (NVU):** Optimalisatie van de *FGFR3* mutatie assay voor de detectie van blaaskanker recidieven o **2010 Postgraduate School of Molecular Medicine, Molecular medicine day 2010 Rotterdam:** Optimalisation of detection of recurrent bladder cancer using a urine-based mutation assay: Poster presentation o **2009 Dutch Urology society (NVU):** *FGFR3* mutatie analyse op urine in de follow-up van patiënten met laaggradig niet-spierinvasief blaaskanker: Presentation o **2009 European Meeting on Molecular diagnostics Scheveningen**: Urine Preservation Medium preserves urinary cells for cytology. Poster presentation o **2009 European Meeting on Molecular diagnostics Scheveningen**: Urine Preservation Medium keeps DNA and Proteins stable for molecular analysis. Poster presentation o **2009 American Urological Association, Annual meeting Chicago**: *FGFR3* mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer. Presentation o **2009 European Association of Urology, Annual meeting Stockholm**: *FGFR3* mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer. Presentation o **2008 Koningin Wilhelmina Fonds scientific meeting, Lunteren**: *FGFR3* mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer. Presentation o **Postgraduate School of Molecular Medicine, Molecular medicine day 2008 Rotterdam:** *FGFR3* mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer: Poster presentation
Awards	**2011 Best Poster presentation: European Association of Urology, Annual meeting Austria:** Optimalisation of detection of recurrent bladder cancer using a urine-based mutation assay. **2011 EPAR PhD student of the year:** Award rewarded to a dedicated researcher with remarkable active social engagement.

Research schools	o **2007- now: PhD member of the Postgraduate School of Molecular Medicine** Aim: reviewing of PhD courses and organizing scientific course/symposia/conferences for PhD students Newly set up courses: Imaging for doctors, Survival analysis for doctors
Extracurricular activities	o **2007- 2010: Member Stichting Projecthulp Suriname** Aim: Voluntary exchange projects between The Netherlands and Suriname: ecological/ educational/social projects. Preparation and guidance of voluteers throughout the project. Fundraising with benefit shows and concerts.

PhD Portfolio

COURSES	ECTS
Basic and Translational Oncology 2006 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**1.8**
Classical Methods for data-analysis, CC02, 2006 Netherlands Institute for Health Sciences	**5.7**
Oncology day: Bladder cancer 2007 Het Nederlands Kanker Instituut - Antoni van Leeuwenhoek ziekenhuis	**0.3**
Clinical and Fundamental Oncology 2007 Nederlandse Vereniging voor Oncologie	**1.6**
Molecular Medicine 2007 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**0.7**
Molecular Diagnostics 2007 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**1.0**
Animal Imaging Workshop by AMIE 2008 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam Applied Molecular Imaging programme at Erasmus MC (AMIE)	**1.4**
Biomedical Research Techniques 2009 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**1.5**
Advanced Imaging techniques for medical doctors 2009 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam Applied Molecular Imaging programme at Erasmus MC (AMIE)	**0.2**
Molecular Diagnostics for Medical Doctors 2010 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**0.2**
Statistics and Survival analysis for Medical Doctors 20120 The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**0.5**
Biomedical English Writing Course for MSc and PhD-students The Erasmus Postgraduate school of Molecular Medicine, Rotterdam	**2.0**
ORAL/POSTER PRESENTATIONS	
2013 European Association of Urology, Annual meeting Milan: Urinary biomarkers in the follow-up of low grade non-muscle invasive bladder cancer patients: Update on the FP7 UROMOL project.	**1.0**
2011 European Association of Urology, Annual meeting Austria: Optimalisation of detection of recurrent bladder cancer using a urine-based mutation assay.	**1.0**
2011 European Association of Urology, Annual meeting Austria: Urinary biomarkers for the detection of non-muscle invasive bladder cancer recurrences: The European UROMOL project.	**1.0**

2011 European Association of Urology, Annual meeting Austria: **1.0**
A urine-based methylation assay for the detection of recurrences during follow-up of non-muscle invasive bladder cancer patients.

2010 Dutch Urology society (NVU): Optimalisatie van de FGFR3 mutatie **0.3**
assay voor de detectie van blaaskanker recidieven

2010 Postgraduate School of Molecular Medicine, Molecular medicine **0.3**
day 2010 Rotterdam:
Optimalisation of detection of recurrent bladder cancer using a urine-based mutation assay:

2009 Dutch Urology society (NVU): FGFR3 mutatie analyse op urine in **0.3**
de follow-up van patiënten met laaggradig niet-spierinvasief blaaskanker

2009 European Meeting on Molecular diagnostics Scheveningen: **1.0**
Urine Preservation Medium preserves urinary cells for cytology.

2009 European Meeting on Molecular diagnostics Scheveningen: **1.0**
Urine Preservation Medium keeps DNA and Proteins stable for molecular analysis.

2009 American Urological Association, Annual meeting Chicago: **1.0**
FGFR3 mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer.

2009 European Association of Urology, Annual meeting Stockholm: **1.0**
FGFR3 mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer.

2008 Koningin Wilhelmina Fonds scientific meeting, Lunteren: **0.3**
FGFR3 mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer.

Postgraduate School of Molecular Medicine, Molecular medicine day **0.3**
2008 Rotterdam:
FGFR3 mutation analysis on voided urine as a strong predictive tool for surveillance of patients with non-muscle invasive bladder cancer:

Josephine Nefkens Institute presentations **7.5**
2007, 2008, 2009

Research group lectures **15.0**
2007, 2008, 2009, 2010

Supervision of students **64.0**

Stephen Tjin,	medical student,	Erasmus University Rotterdam
Cheno Abas,	medical student,	Erasmus University Rotterdam
Willemien Beukers,	medical student,	Erasmus University Rotterdam
Jeannine Refos,	biomedical student,	Leiden university medical center
Serdar Yavuzyigitoglu,	medical student,	Erasmus University Rotterdam
Annemieke Nieuwboer,	medical student,	Erasmus University Rotterdam
Jie-Fen Tjin,	medical student,	Erasmus University Rotterdam
Aleksander Herzegovac,	medical student,	Erasmus University Rotterdam