A valuable pair - candidate biomarkers RBM3 and PODXL in urothelial bladder cancer

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A valuable pair

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PODXL in urothelial bladder cancer

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Urothelial bladder cancer is a heterogeneous disease with very different outcomes for patients. Current prognostic tools hold room for improvement and predictive tools are largely missing. Karolina Boman is a medical oncologist who during her doctoral studies has examined the prognostic and predictive value of the candidate biomarkers PODXL and RBM3 in urothelial bladder cancer. The results and potential implications for improved patient care are presented in this thesis.

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A valuable pair – candidate biomarkers RBM3 and PODXL in urothelial bladder cancer

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Title: A valuable pair: Candidate biomarkers RBM3 and PODXL in Urothelial Bladder Cancer

Abstract: Bladder cancer is a heterogenous disease, ranging from minimally invasive, low-grade tumours with low recurrence rates and mortality on one end of the spectrum, and muscle invasive, high-grade disease prone to recurrence, progression and death at the other end.

The aim of this thesis was to investigate the expression, clinicopathological correlates and prognostic significance of the candidate biomarkers podocalyxin-like protein (PODXL, papers II and III) and RNA-binding motif protein 3 (RBM3, papers I, III and IV) in urothelial bladder cancer (UBC). In paper IV, the potential predictive significance of RBM3 was also examined. The candidate biomarkers were examined alongside established clinical risk factors.

RBM3 expression was evaluated by immunohistochemistry in tissue microarrays (TMA) from three different patient cohorts (n=343 in paper I, n=272 in paper III and n=151 in paper IV). In paper I, negative RBM3 expression was significantly associated with unfavourable tumour characteristics and was an independent predictor of shorter disease-specific survival (DSS) as well as 5-year overall survial (OS). Patients with Ta/T1 tumours displaying negative RBM3 expression had a significantly reduced 24 month progression-free survival (PFS) and 5-year OS. No association was seen between RBM3 expression and recurrence. In paper 3, these associations were validated, although with a somewhat different cut-off. Low RBM3 expression was significantly associated with unfavourable tumour characteristics and was an independent predictor of a shorter OS in both the full cohort and in T1 disease.

In paper IV, the expression of RBM3 was evaluated in tumours from 151 patients treated with cystectomy due to muscle-invasive UBC, 45.7% of which had received neoadjuvant chemotherapy (NAC). RBM3 expression was not prognostic in the full cohort. However, when accounting for NAC, there was a significantly reduced RFS in in the group of patients with high RBM3 expression who had not been treated compared to those that had received NAC (p=0.044). The association between high RBM3 expression and response to chemotherapy was strengthened by the silencing of RBM3 in UBC cell lines, rendering them less sensitive to cisplatin and gemcitabine.

PODXL expressed in the cell membrane was evaluated by immunohistochemistry in TMA from three different patient cohorts (n=100 and n=343 in paper II and n=272 in paper III). Membranous expression of PODXL was strongly and significantly associated with unfavourable tumour characteristics in all three cohorts. In paper II, PODXL independently predicted a shorter DSS and OS in the full cohort, and a shorter PFS and DSS in patients with Ta/T1 tumours. In paper III, membranous PODXL expression was significantly associated with a shorter OS in both the full cohort and T1 tumours, but not independent of other prognostic factors.

The conclusions drawn from these studies are that both RBM3 and PODXL are potentially clinically useful biomarkers in UBC. RBM3 may have clinical implications in NMIBC for decision making in the pre-cystectomy setting and for its predictive value in patients under consideration for NAC. PODXL is associated with an adverse prognosis, making it a potentially useful prognostic biomarker. Both candidate biomarkers show great promise, although their value should be further examined in a prospective setting.

Key words: Urothelial bladder cancer, RBM3, PODXL, prognosis, prediction, biomarker, immunohistochemistry, IHC, tissue microarray, TMA

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Coverphoto "Bladder cherries" by Helhet Reklam

"If you have a brain, you are obliged to use it."

-Meryl Streep

Content

Abbreviations

CI	Confidence interval
DFS	Disease free survival
CIRP	Cold inducible RNA-binding protein
CSS	Cancer specific survival
DFS	Disease free survival
DSS	Disease specific survival
CT	Computed tomography
DNA	Deoxyribonucleic acid
EMT	Epithelial to mesenchymal transition
EORTC	European Organization for Research and Treatment of Cancer
EAU	European Association of Urology
IHC	Immunohistochemistry
MDCS	Malmö Diet and Cancer Study
MIBC	Muscle-invasive bladder cancer
mRNA	Messenger RNA
NMIBC	Non muscle-invasive bladder cancer
OS	Overall survival
PFS	Progression free survival
PODXL	Podocalyxin-like protein 1
PUNLMP	Papillary urothelial neoplasm of low malignant potential
RBM3	RNA-binding motif protein 3
RNA	Ribonucleic acid
RFS	Recurrence Free Survival
siRNA	Small interfering RNA
TMA	Tissue microarray
TNM	Tumour/Nodes/distant Metastasis
UBC	Urothelial bladder cancer
WHO	World Health Organization

List of publications

This thesis is based on the following publications, which are referred to in the text with roman numerals:

I. Boman K, Segersten U, Ahlgren G, Eberhard J, Uhlén M, Jirström K, Malmström PU. Decreased expression of RNA-binding motif protein 3 correlates with tumour progression and poor prognosis is urothelial bladder cancer. *BMC Urology* **13**:17 (2013)

II. Boman K, Larsson AH, Segersten U, Kuteeva E, Johannesson H, Nodin B, Eberhard J, Uhlén M, Malmström PU, Jirström K. *British Journal of Cancer* **108**(11):2321-2328 (2013)

III. Boman K, Andersson G, Wennersten C, Nodin B, Ahlgren G, Jirström K. Podocalyxin-like and RNA-binding motif protein 3 are prognostic biomarkers in urothelial bladder cancer: a validatory study (submitted)

IV. Boman K, Karnevi E, Nodin B, Ahlgren G, Jirström K. Translational study of RBM3 as a biomarker of response to neoadjuvant chemotherapy in urothelial bladder cancer (manuscript)

Introduction

Cancer

Cells in the body are normally under strict control. They divide and differentiate to serve the organism as a whole. Cancer is when cells acquire changes in their genes, i.e. mutations, which enable them to disrupt this control. Thus, all cancer is genetic.

The changes that pave the road from a normal cell to a metastatic cancer are complex, but six hallmarks of cancer were proposed in a paper from 2000 (1), and has since been one of the most quoted papers in oncology, with almost 25000 citations to date. These proposed hallmarks consist of the features of resisting cell death, sustaining proliferative signalling, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis. In 2011, an updated article on the hallmarks of cancer was published, where the authors proposed genomic instability as an underlying factor for the acquisition of the previously described hallmarks, and inflammation as a contributing factor. Two new features, with a growing body of data, were proposed as emerging hallmarks: reprogramming of cell metabolism and evading the immune system (2). All these features together contribute to the evolutionary process that is cancer. The expression "survival of the fittest" also applies to cancer cells; with cells fostering alterations that confer the capability to proliferate and survive gaining an advantage over other cells (3).

Tumour suppression

As previously mentioned, the body's cells are normally rigorously controlled and many checkpoints are needed before a cell is allowed to divide. Tumour suppressor genes, or to be more precise, the proteins for which they code, have a negative effect on the cell cycle or promote apoptosis and make out an important part of powerful programs that regulate cell proliferation (2). Tumour suppressors govern a wide range of cellular activities in the normal cell. Even though their key function is not to protect individuals against cancer, their role in cell cycle

checkpoint control, mitogenic signalling pathways, DNA-damage and other stress-response functions illustrates the complexity of cell-autonomous processes that can be affected in in cancer cells. These tumour suppressors have often been identified as such in animal models, with individuals with loss of normal function being at higher risk of developing malignancies. Germline mutations in important tumour suppressor genes are also the cause of certain syndromes in humans associated with an increased risk of malignancy, often at a younger age and with multiple primary tumours, underscoring the importance of their protective role in the normal cell. Such syndromes include Li-Fraumeni (4), hereditary breast cancer (5) and Lynch Syndrome (6).

Biomarkers

According to the National Institute of Health Biomarkers Definitions Working Group, the term biomarker can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (7) In oncology, a biomarker can be used as:

1. A means of screening a population

2. A diagnostic tool

3. A prognostic marker, which indicates the likelihood of a specific patient outcome

4. A predictive marker, which indicates the likelihood of benefiting from a specific therapy or

5. An indicator of tumour regression or progression, typically a blood test

Though a large amount of published papers on cancer biomarkers exists, only a few have made it into the clinic. This may be due to the difficulties in reproducibility between studies (8, 9) and different methodological aspects have been cited to explain these discrepancies. It may also be due to the high performance specifics needed for any biomarker to be clinically useful and motivate its transition "from bench to bedside" (10).

Figure 1. Challenges and suggested focus areas for different sides of the implementation of biomarkers (11).
Reprinted with permission from the American Society for Biochemistry and Molecular Biology.

In all the different areas of UBC, there is a need for novel biomarkers. This includes those that could be useful in:

1. Early detection of the disease

2. Staging and monitoring

3. Determining the prognosis

4. Prediction of response to therapy and

5. Monitoring disease progression

To develop biomarkers in any of these areas could help in the efforts of reducing bladder cancer morbidity and mortality and help the field of personalized cancer care (12).

REMARK criteria

In 2005, McShane et al presented a paper in the British Journal of Cancer (8), were the abstract stated, "Despite years of research and hundreds of papers on tumour markers in oncology, the number of markers that have emerged as clinically useful is pitifully small". Some of the problems stated by this group, and others, are general methodological differences, poor study design, assays that are not standardized or lack reproducibility, and inappropriate or misleading statistical

analyses often based on sample sizes too small to draw meaningful conclusions. In an effort to change this situation, this paper presented reporting guidelines for a more homogenous and reproducible manner of planning and presenting studies on prognostic biomarkers. The guidelines are shown in the Table 1, and include precise details on materials and methods, with guidelines on which characteristics and techniques should be included in the manuscript for transparency and reproducibility.

Table 1. Reporting recommendations for tumour marker prognostic studies Reprinted with permission from the Nature Publishing Group (8).

Guidelines for the REporting of tumor MARKer studies (REMARK)

Introduction
    State the marker examined, the study objectives, and any prespecified hypotheses

Materials and methods
    Patients
        Describe the characteristics (e.g., disease stage or comorbidities) of the study patients, including their source and inclusion and exclusion criteria
        Describe treatments received and how chosen (e.g., randomized or rule-based)
    Specimen characteristics
        Describe the type of biological material used (including control samples) and methods of preservation and storage
    Assay methods
        Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study end point
    Study design
        State the method of case selection, including whether the study design was prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time
        Precisely define all clinical end points examined
        List all candidate variables initially examined or considered for inclusion in models
        Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size
    Statistical analysis methods
        Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled
        Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination

Results
    Data
        Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events
        Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values
    Analysis and presentation
        Show the relation of the marker to standard prognostic variables
        Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan–Meier plot is recommended
        For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model
        Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance
        If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation

Discussion
    Interpret the results in the context of the prespecified hypotheses and other relevant studies; include a discussion of limitations of the study
    Discuss implications for future research and clinical value

Urothelial Bladder Cancer

A hallmark of UBC is its variable prognosis. Out of non muscle-invasive tumours, 70% will recur and 10-20% will become muscle-invasive (13). Tumours that are muscle-invasive have a high risk of progression into subsequent metastatic disease, a deadly disease (14).

Anatomy, histopathology and staging

The normal bladder is a reservoir for urine and consists, from the inside out, of the urothelium, the lamina propria, the muscularis propria (the detrusor muscle) and the perivesical fat. Bladder cancer staging follows these layers, with increasing T-stage with increasing depth of invasion. Tumour stages Tis and Ta are confined to the mucosa and T1 an invasion into the lamina propria is seen, but these T-stages are defined as non muscle-invasive (NMIBC). Stages T2 to T4 are muscle invasive, with varying degrees of invasion into the muscle layer and beyond (Figure 2a).

The pathological diagnosis of bladder cancer is most often made by transurethral resection of the bladder (TURB). This procedure often has multiple goals; to remove the tumour, to clinically stage the tumour and to obtain tumour tissue for histopathological evaluation.
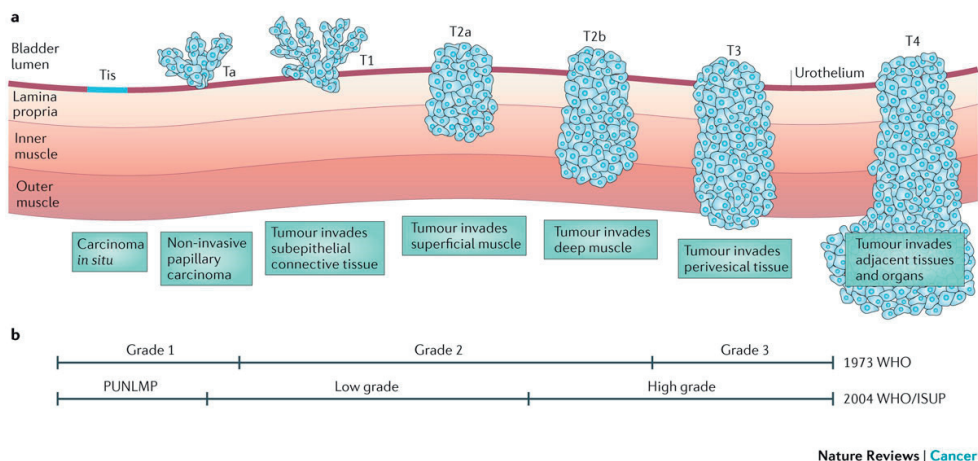
Figure 2a. An illustration of different T-stages in UBC and 2b. an overview of the differences between the WHO 1973 and the WHO/ISUP 2004 classifications. Reprinted with permission from Macmillan Publishers Ltd (15)

The histopathological grading is determined by the microscopic appearance of the tumour cells and is done according to the World Health Organization (WHO)-classification (16). The initial WHO classification of 1973 had a high interobserver variability and lacked clear definitions of the three pathological grades, with increasing percentage of tumours classified as grade 2, rendering this group excessively large (17-20). The 2004 classification from WHO/ISUP (International Society for Urological Pathology) specified detailed histological criteria to reduce interobserver variability (21). With the 2004 classification, the distinction between non-muscle invasive and muscle-invasive disease was stressed, and the term "superficial" bladder cancer omitted. The European Association of Urology (EAU) guidelines for NMIBC recommends the simultaneous use of the WHO classifications of 1973 and 2004, as the 2004 classification has not yet been incorporated into validated prognostic models (22). A simplification of the differences between the 1973 and 2004 classifications is illustrated in Figure 2b.

Bladder cancer may present with several "variant" histologies, i.e. unusual morphological features that deviate from the conventional appearance. These variant histologies seem to be underrecognized in the clinical setting (23), and include a large variety of different subtypes, frequently associated with a poor prognosis (24) and upstaging at the time of cystectomy (25).

Epidemiology and etiology

Urothelial bladder cancer (UBC) is a disease that arises in the urothelium, i.e. the transitional epithelium, the inner lining of the urinary tract. Urothelial carcinoma is the most common histologic type in Europe and the United States, representing around 90% of bladder cancer cases (26). The most common symptom of UBC is hematuria, but the initial symptoms can also be irritative and obstructive voiding symptoms such as frequency, urgency or dysuria. As these types of symptoms are common, most patients seek the attention of the health care system and subclinical disease seems to be rare; in an autopsy study performed on 3118 individuals in the USA, evaluating the frequency of undiagnosed malignant tumours at the time of death, only two cases of UBC were found (27).

UBC is a disease affected by age and the surrounding environment. The incidence and mortality rates of the disease vary by country, ethnicity, sex and age (28). UBC is more common in older people, with a median age of 73 years at diagnosis (29). Globally, UBC is the ninth most common cancer with 430,000 new cases diagnosed in 2012 and it is the most common malignancy involving the urinary system (30, 31). In 2012, there were an estimated 118,000 cases and 52,000 deaths due to bladder cancer in Europe (14).

UBC is the 7th most common cancer in men and the 17th most common cancer in women worldwide (28). As depicted by these statistics, men have a higher UBC incidence of up to three or four times that of women (32, 33). This may be due to higher smoking rates and occupational hazards in the male population, although differences in metabolic enzyme activity and sex steroids are believed to play a part (33, 34), as portrayed in Figure 3.

Reaction with nucleophiles in the
liver & potential liver damage

↑

Highly reactive metabolites
(.e.g. sulfate conjugates)

Androgen ⊣ ↑ ◄ **Estrogen**

Ar☐NH$_2$ metabolism in the **Liver**

Androgen → ↓ ⊢ **Estrogen**

Metabolites with relatively low
reactivity (e.g. glucuronides)

↓

Urinary excretion & conversion
to carcinogenic species

↓

Bladder damage

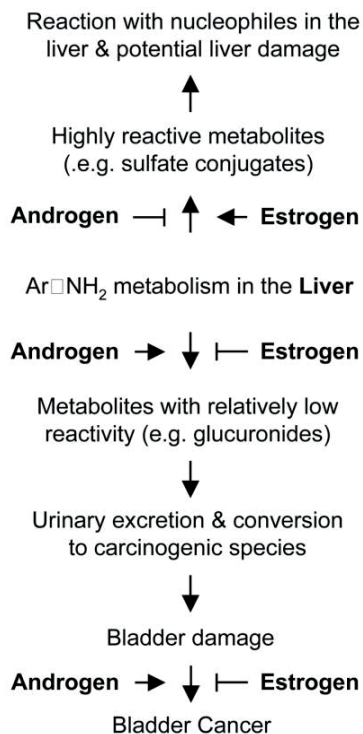**Androgen** → ↓ ⊢ **Estrogen**

Bladder Cancer

Figure 3. The potential interplay between sex steroids and the liver, which may be responsible for the gender disparity in UBC. The illustration shows the potential opposing effects of androgen and estrogen on liver handling of aromatic amines and on carcinogenesis in the bladder (34). Reprinted with permission from PMC Publishing.

Although the incidence is lower among women, most studies show that female sex is correlated to more advanced disease at diagnosis and reported, though not universally, to be associated with higher risk of disease recurrence, progression and disease specific death (29, 33).

As with all cancer, the human toll is enormous. With UBC, the economic costs are as well. High recurrence rates and a monitoring program that requires invasive procedures are likely contributors (35). In the US, bladder cancer has the highest lifetime cost per patient, and the fifth overall highest cost (36, 37).

Tobacco smoking is the most common risk factor for UBC, accounting for approximately half of all cases (28). It has been confirmed that tobacco smoking also increases the risk of tumour recurrence and progression (38, 39). Hence, cessation of smoking is important both as a primary as well as a secondary intervention against UBC. Individuals exposed to aromatic amines, polycyclic aromatic hydrocarbons and chlorinated hydrocarbons, mainly occupational exposure in industrial settings, also have an increased risk of developing UBC (28,

40). The risk of developing UBC when exposed to risk factors is also dependent of genetic predisposition (28, 41).

Alterations in bladder cancer

As previously discussed, development of cancer is a process where fundamental cell functions are disrupted, leading to a malfunction in cell regulation (1). Two well-investigated genetic alterations in UBC are the tumour suppressor gene TP53 and the oncogene FGFR3. TP53 mutations are common in high-grade, high-stage tumours and are rarely seen in low-grade, low-stage tumours (42, 43). FGFR3 mutations are found in both low-grade, low-stage tumours as well as high-grade, high-stage disease, though this mutation is rarely seen together with mutations in the TP53 gene. It has been proposed that UBC has two diverging pathways leading up to either NMIBC or MIBC, with one path driven by an increase of oncogenes, often preceded by hyperplasia, and the other by a decrease in tumour suppression, preceded by CIS or hyperplasia (44), Figure 4. The observation that the genetic alterations in dysplasia and CIS correspond to those found in high-grade tumours strengthens this notion (44).
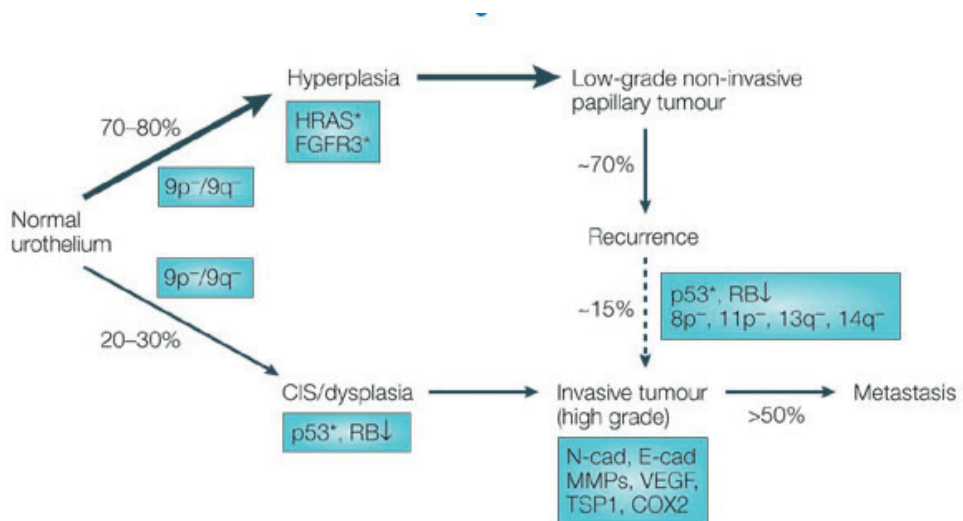

Figure 4. Genetic and epigenetic defects that characterize the divergent pathways of urothelial tumorigenesis. Reproduced with permission from Nature Publishing Group (44).

Many groups have made efforts to characterize UBC molecularly based on gene expression and multiple independent, and in many cases overlapping, systems have evolved. A schematic overview of the systems presented by the groups at the University of North Carolina, the MD Anderson Cancer Centre, the Cancer Genome Atlas and the Lund Bladder Cancer group is presented in Figure 5.
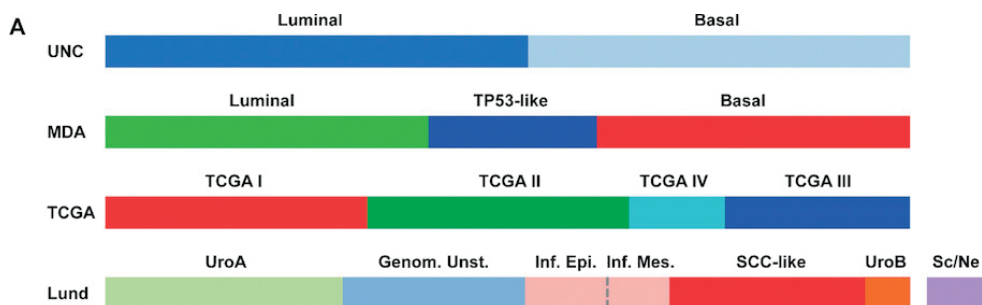
Figure 5. Schematic representation of subtype classification overlaps between the systems developed by groups at the University of North Carolina (UNV), the M.D. Anderson Cancer Centre (MDA), the Cancer Genome Atlas (TCGA) and Lund group (45). Reprinted with permission from Elsevier Publishing.

The Lund Bladder Cancer Group was the first to characterize UBC based on gene expression, and has done so with both MIBC and NMIBC tumours. They reported five subtypes; urobasal A (UroA), urobasal B (UroB), genomically unstable (GU), squamous cell carcinoma like (SCCL) and an infiltrated tumour class (46). The different groups had different prognoses. Efforts to find immunohistochemical surrogate markers to the different subgroups have however not yet been entirely successful (47).

Tumour suppressors in UBC

P53 was dubbed as the "guardian of the genome" by Lane et al in 1992 (48) and as the "cellular gatekeeper" by Levine et al in 1997 (49). It is one of the most well known and well researched tumour suppressors (50). P53 is a protein that, when activated, orchestrates the cell's response to different stress signals such as DNA-damage, oncogene activation, hypoxia, cellular starvation and oxidative stress.

The activation of p53 is initiated when the interactions between p53 and the negative regulators MDM2 and MDM4 are disrupted (51, 52), allowing p53 to act

as a transcriptional factor, bind to DNA, initiate the transcription of target genes with responses that include transient cell cycle arrest, cellular senescence (permanent cell cycle arrest) and apoptosis (53, 54). MDM2 and MDM4 also play a part in regulating the subsequent degradation of p53 (55).
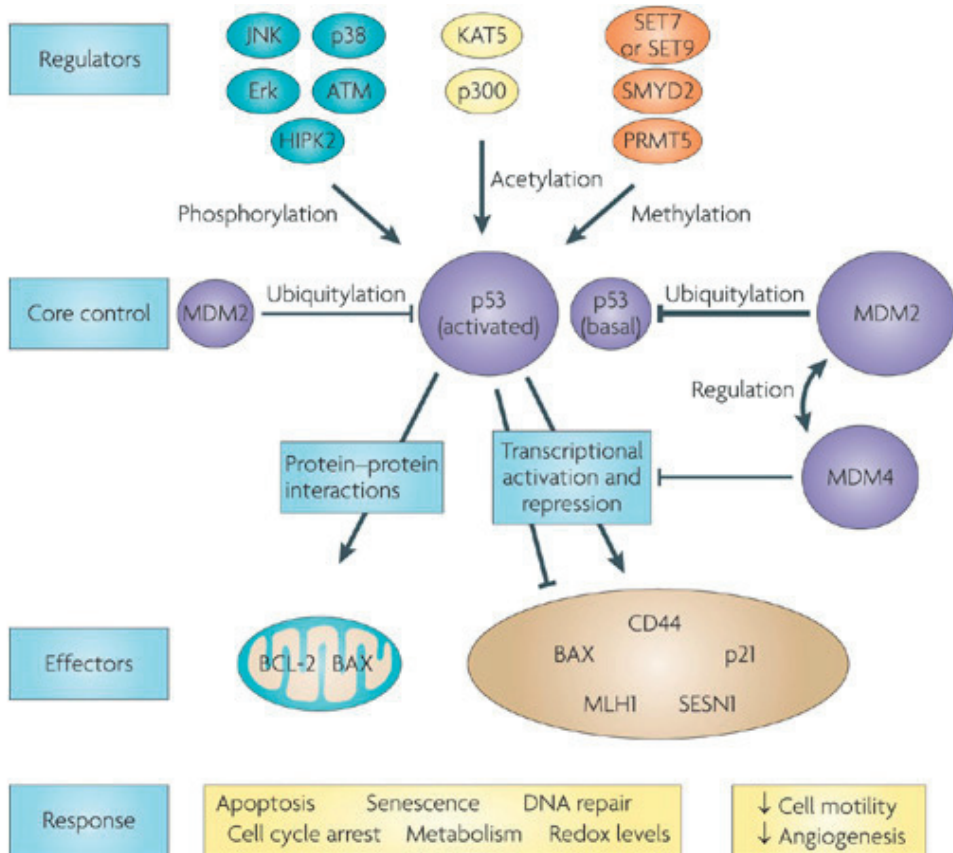
Figure 6 Overview of the regulators, core control, effectors and response of p53 (56). Reprinted with permission from the Nature Publishing Group.

Halting a damaged cell's progression through the cell cycle until repaired and inducing apoptosis in those with irreparable damage promotes cellular and genetic stability and lies at the heart of successful tumour suppression (48). TP53, the gene encoding the protein p53, is a gene that is frequently mutated in UBC, especially

in high-stage, high-grade disease (42). However, it has not been proven to be useful in the prediction of progression into muscle invasive disease in prospective studies (57, 58).

Another tumour suppressor that has been found to be affected in UBC is the Retinoblastoma gene (Rb), which encodes the protein pRb. This protein sequesters the powerful transcription factor E2F (59), the products of which target genes in turn facilitate the G1/S transition and S-phase. In the normal cell, growth factor stimulation via the MAPK/ERK pathway induces the phosphorylation of pRb by cyclin D/cdk4 and cyclin E/cdk2 to subsequently release E2F. The cyclins are in turn regulated by p16 and p27 (60). Changes in all these steps of the cell cycle regulation have been seen in UBC, with loss or hyperphosphorylation of Rb, overexpression of cyclin D and E and their partners cdk2 and cdk4, and underexpression or loss of p16 and p27 (61).

Oncogenes in UBC

The oncogene fibroblast growth receptor 3 (FGFR3) is a protein tyrosine kinase growth factor receptor that is frequently point mutated in NMIBC (60-70%), and less frequently so in MIBC (5-20%) (15). It has been linked to an improved survival in T1 UBC (57). However, and importantly, in the small subset of MIBC that has FGFR3 mutation, frequent deletion of the CDKN2A gene, encoding the tumour suppressor p16, is found. Hence, this may represent a progression pathway for NMIBC into MIBC (62).

Another oncogene implicated in the tumorigenesis of UBC, especially that of NMIBC, is HRAS. It has been demonstrated to be mutated in UBC, mainly in NMIBC, but this mutation and FGFR3 mutations are mutually exclusive, suggesting alternative routes that lead up to the same phenotype (63). The role of HRAS in UBC has been tested in animal models, where mice with activating HRAS mutation developed urothelial hyperplasia early on and papillary NMIBC in the later stages of life, indicating that HRAS requires additional mutations to establish a tumour (64).

22

Treatment

Non-muscle invasive disease

The initial management of presumed UBC is a transurethral resection of all visible bladder tumours (TURB), including any areas of suspected carcinoma in situ (CIS) and with adequate resection depth to allow the assessment of invasion of the tumour into the muscularis propria. An examination under anaesthesia should also be performed since induration or a palpable mass suggest invasion into the muscle or beyond and appropriate diagnostic imaging performed to evaluate if additional tumours are present in the urinary system, either seen as filling defects or hydronephrosis (22). This examination, together with the histopathological examination, will decide the subsequent therapy, if any. Residual tumour after the first TURB has been observed in 33-71% of patients with T1 tumours and in 41.4% of TaG3 tumours (65, 66). To find any residual cancer, repeat TURB is now standard of care in all cases of high-grade NMIBC (22). The risk that a T1 tumour has been understaged and is *de facto* muscle-invasive in the repeat TURB ranges from 4 to 25% (67), stressing the importance of a well performed initial TURB, as well as that of a repeat TURB. Improved detection of recurrences can also be obtained by the use of Hexvix (hexaminolevulinat) and blue-light cystoscopy (68).

NMIBCs are subdivided into three stages based on their growth pattern and depth of invasion, Ta, Tis/CIS and T1 lesions. This together with grade, number of tumours, the frequency of recurrence and concomitant CIS will guide the clinical management (22). The two models most often used for prediction of the risk of recurrence and progression are the validated risk tables by the European Organisation for Research and Treatment of Cancer (EORTC) (69) and by the Club Urologico Español de Traitamiento Oncológico (CUETO) (70).

The EORTC scoring system is based on a study population of 2,596 patients with NMIBC from seven different EORTC trials. Repeat TURB was not performed, nor was maintenance BCG given. Seventy-eight percent of the patients received intravesical therapy, predominantly with chemotherapy. The scoring system has four risk categories for recurrence and for progression, based on the six most significant clinical and pathological factors: number of tumours, tumour size, prior recurrence rate, T-category, presence of concomitant CIS and tumour grade according to WHO 1973 (69).

The CUETO scoring system was developed based on a total of 1,062 patients from four CUETO studies treated with maintenance BCG. Repeat TURB was not performed. The risk score for recurrence is based on sex, age, grade, tumour status (i.e. recurrent or primary), multiple tumours and concomitant CIS. For progression, the variables were age, grade, T-stage, number of tumours and concomitant CIS (70). The CUETO tables yield a lower calculated risk of recurrence than the EORTC tables, probably due to the effects of BCG (70).

Validating studies of these risk tables have reported some overestimation regarding both the risk of recurrence and progression and, although clinically useful, the differences between the cohorts used and the clinical population may affect the risk estimation based on these tables (71-73).

Following proper staging, NMIBC cases will be treated according to their respective risk category with or without intravesical instillation of chemotherapy or Bacillus Calmette-Guérin (BCG).

Intravesical chemotherapy with one dose of chemotherapy, typically epirubicin or mitomycin C immediately after TURB may be administered to reduce seeding and re-implantation of residual tumour cells in the bladder, especially at the site of resection (74, 75). In low and intermediate risk Ta and T1 tumours with previous low recurrence rates (≤1 recurrence per year) this has been shown to prevent recurrences and has few side effects (76-78).

Bacillus Calmette-Guérin has since its introduction in the 1976 been a cornerstone in the adjuvant treatment of NMIBC (79). In a meta-analysis published in 2001, BCG decreased tumour recurrence by 43% compared to 16 to 21% with intravesical chemotherapy (80). BCG is considered standard of care for patients with intermediate- and high-risk NMIBC, i.e. patients with features such as the presence of CIS, high-grade Ta tumours and T1 tumours (22). However, despite its lengthy clinical use, the understanding of its mechanism of action still contains gaps. It does however seem to stimulate both the innate and the adaptive immune system after administration, as portrayed in Figure 7. Once instilled into the bladder, BCG attaches to fibronectin, a glycoprotein that mediates cell adhesion, and is thereafter internalized into cells. This internalization initiates a cascade of inflammatory response that promotes cell-mediated antitumour activity, conveyed by CD8+ T-cells, natural killer (NK)-cells, macrophages and granulocytes (81).

The immune reaction that is evoked by BCG is evident through the mass of cytokines transiently secreted into the urine, including interleukin (IL)-1, IL-2, IL-5, IL-6, IL-8, IL10, IL-12, IL-15, IL-18, interferon-inducible protein (IP)-10, tumour necrosis factor (TNF)-α, granulocyte-monocyte colony stimulating factor GM-CSF) and interferon (IFN)-γ (82).
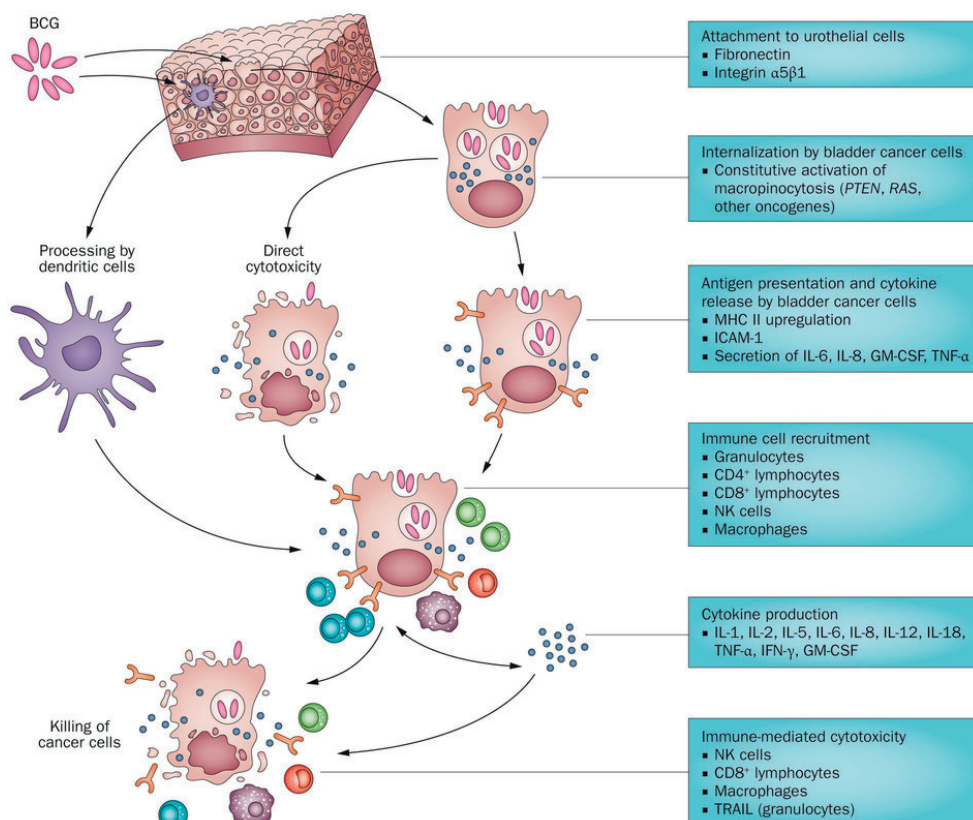
Figure 7. According to this model, live BCG attaches to the urotelium via fibronectin and integrin α5β1, and is internalized by bladder cancer cells, owing to oncogenic aberrations that activate macropinocytosis. Following internalization, bladder cancer cells upregulate expression of MHC class II and ICAM-1, and secrete cytokines that, along with dendritic cells, recruit immune cells to the site, resulting in cytotoxicity to bladder cancer cells, proceeding through various immune mechanisms (81).

Muscle-invasive disease

Patients with UBC invading the detrusor muscle, i.e. T2 tumours and beyond, will in approximately 50% of cases eventually die from metastatic disease, despite definitive local therapy with cystectomy and/or local radiotherapy. In a large series of patients with MIBC treated with radical cystectomy with radical lymphadenectomy (n=507) who were not treated with neoadjuvant chemotherapy (NAC) and with preoperative node-negative (N0) staging, the investigators found lymph node metastases in 24% of the patients. This number shows the metastatic

capability of UBC. RFS was 62% at 5 years for the entire group and in patients with N0, organ-confined (<T3) disease, the corresponding number was 73% (83).

In an even larger cohort of patients treated with radical cystectomy and lymphadenectomy (n=1,054), though some treated with adjuvant chemotherapy or radiotherapy, recurrence free survival at five years was 68% for the full cohort, and the same number for T2, N0 tumours was 89%. The majority of deaths due to UBC occurred within the first three years of follow-up after cystectomy. According to the conclusions drawn by the authors, only patients with N+ disease had a survival benefit from adjuvant therapy (84). Perioperative deaths in both series of patients were approximately 3% (83, 84).

Neoadjuvant therapy with cisplatin-based combination therapy is recommended for patients with MIBC in the EAU guidelines, if otherwise medically fit (85). This recommendation is based on several trials evaluating the potential survival benefit of NAC. The results from these studies were evaluated in a large meta-analysis published in 2005, where pooling of 3005 patients from 11 trials was performed. This constituted 98% of all patients from eligible randomised controlled trials. The authors found a significant benefit regarding OS (HR; 0.86, 95% CI 0.77-0.95) in favour of cisplatin-based combination NAC, the equivalent of a 5% survival benefit after 5 years. The authors also found a significantly prolonged DSS with cisplatin-based combination NAC (HR 0.78, 95% CI 0.71-0.86), equivalent to 9% absolute survival benefit at 5 years, and hence postulated that the use of cisplatin-based combination chemotherapy should be the golden standard to which other therapeutic regimens should be judged (86). There is however an unmet need for biomarkers that can predict response to chemotherapy. A gene expression model proposed to predict response to NAC has been proposed by Takata et al, but has only been evaluated in small cohorts and lacks validation (87, 88).

The use of adjuvant chemotherapy is under debate. The European Association of Urology (EAU) has pointed out that the studies evaluating the potential benefit of such treatment have problems with inadequate power, poor accrual and flaws in study design and statistical analysis (89). The results of the so far largest randomized controlled trial (EORTC 30994), demonstrated a significant improvement in PFS (HR 0.54, 95% CI 0.40-0.73) although it failed to show a significant difference in OS (90). A meta-analysis including 945 patients from nine trials demonstrated an improved OS with adjuvant treatment (HR 0.77 95% CI 0.59-0.99), although the pooled trials were all afflicted with flaws regarding study quality (91). Similar results regarding OS were demonstrated in a large retrospective cohort from 11 centres including 3,947 patients (HR 0.83, 95% CI 0.72-0.97) (92). The EAU recommendation is, in light of these results, that adjuvant, cisplatin-based adjuvant chemotherapy can be considered for patients

with pT3/T4 and/or pN+ disease who have not received NAC, although the level of evidence is still poor (89).

Metastatic disease

When a UBC has spread and the metastasis is not available for curative surgery or radiotherapy, lasting complete remissions are few and far between. Multiple studies have shown that UBC is generally sensitive to chemotherapy, with objective response rates of up to 65-75% with combination regimens (93). However, the disease is prone to gain resistance mechanisms and patients subsequently progress during chemotherapy.

The combination of methotrexate/vinblastine/doxorubicin(adriamycin)/cisplatin (MVAC) is considered a standard first-line option based on several randomized trials. Loehrer et al published the results of a multicentre trial of 269 patients with advanced UBC randomized either to single agent cisplatin or MVAC. Results showed a significant improvement in overall response rates (39 vs. 12 percent), a significantly longer PFS (10 vs. 4 months) and a significant improvement in OS (13 vs. 8 months). A major concern of MVAC is however the toxicity. In a series of patients treated with MVAC in 1989, the investigators found a hospitalization rate of 54% (94). This was however before some of the drugs used to ameliorate the side effects of the treatment used today were introduced, not least modern anti-emetics and granulocyte colony stimulating factor (G-CSF), making the authors' concluding remark "*...our results suggest caution in the widespread application of this protocol.*" somewhat out of date. The side effects of MVAC include myelosuppression, neutropenic fever and infection, mucositis as well as nausea and vomiting. Deaths related to toxicity have been reported in most trials including MVAC to patients with UBC (94-96).

In 2005, von der Maase et al. published a long term follow-up from a trial randomizing patients with locally advanced or metastatic UBC to treatment with either gemcitabine/cisplatin (GC) or MVAC, n=405. Both OS and PFS were similar, with a median survival of 14.0 months for patients receiving GC and 15.2 months for patients receiving MVAC (97). GC has also been shown to have a superior safety profile, with more patients completing 6 cycles of therapy, with fewer dose adjustments, less therapy-associated deaths than MVAC (1% vs. 3% deaths) and less grade 3 and 4 toxicity (including neutropenia, neutropenic sepsis and mucositis), as well as a well-maintained quality of life, but with GC patients scoring better regarding maintained weight, performance status and fatigue (98). The combination could thus also be considered a standard first-line regimen, even though the von der Maase study was not designed to demonstrate comparable effect of the two regimens. Although the survival with a modern chemotherapy

regimen is superior to the three month survival after the onset of metastasis seen in historical data (99), the five-year survival rate was only shown to be approximately 15% (97).

The effect of addition of paclitaxel to GC (PGC) compared to GC as first line treatment was evaluated in 626 patients by the EORTC30987 study. Final results showed an increased objective response rate (ORR) (56 vs. 44%) and a trend towards an improved PFS and OS in the full cohort, but with higher rates of grade 3 and 4 toxicity (100).

For patients deemed unfit for cisplatin-based therapy, carboplatin-based regimens can be considered if they are otherwise fit for chemotherapy. The EORTC30986 trial compared the OS between chemo-naive patients with either renal impairment or PF=2, randomly assigned to either gemcitabine plus carboplatin (GC) or to methotrexate, carboplatin and vinblastine (M-CAVI). The final results of this trial suggested that the different regimens were equally effective, but with less grade 3 and 4 toxicity in patients treated with GC (101).

A new, and very promising, addition to the treatment arsenal for advanced UBC is that of immunotherapy, with several trials testing checkpoint inhibition in the metastatic UBC setting (102).

RBM3

The central dogma of biology is that the flow of genetic information is unidirectional and leads away from the DNA, or in simplified terms; "DNA makes RNA and RNA makes protein". This linear description has evolved since it was first mentioned in 1958 (103), not in least the part of "DNA makes RNA". The process starts with DNA, with a specific gene sequence being transcribed into a first template, the preRNA. The preRNA subsequently undergoes posttranscriptional modifications, mediated by small RNAs and different RNA-binding proteins (RBPs). RBPs contain one or more RNA binding domains/motifs (RBDs). These RBDs have a broad repertoire of actions exerted on their target mRNA, and much is yet to explore about these molecules and their effect on posttranscriptional modification. Around 40 RBDs have been identified and some of the best described RBDs are the RNA recognizing motif (RRM), K-homology domain (KM) and zinc finger (104).

After these modifications a functioning messenger RNA-template (mRNA) can be transported out of the nucleus, into the cytoplasm, where it can be translated into the sequence of amino acids that will form the protein. The process of translation will take place on a ribosome, to which transfer RNA (tRNA) will carry the amino acids needed. The protein undergoes quality control and is folded into its final three-dimensional structure (105).

Function in the normal cell

RNA binding motif protein 3 (RBM3) contains an RNA recognizing motif (RRM), i.e. a distinct sequence that has a three-dimensional structure that allows binding to RNA. The RRM in turn contains ribonucleoprotein domains (RNPs). The RNPs (RNP1 and RNP2) in RBM3 show sequences and functions related to parts of cold shock proteins that are evolutionarily conserved between species. The RRMs in RBM3 can also bind to DNA, and thus affect transcription (106). The gene encoding RBM3 was discovered in 1995 by Derry et al (107) and its location was mapped to the p11.23 region of the X-chromosome.

RBM3 belongs to a group of stress-response proteins with a similar RNA-binding domain at the N-terminal, and increases in response to various endogenous and environmental stress factors (108), including mild to moderate hypothermia (109). It is known to modulate the translational process in multiple manners. Generally, when upregulated, RBM3 enhances global protein translation by interactions with the ribosomal subunits and initiation factors (110, 111). At the same time, RBM3 has been found to enhance the levels of many micro-RNAs (miRNA), small non-coding RNA molecules that are involved in the process of the degradation of their target mRNAs. This would however seem contradictory to the role of RBM3 in an increased protein synthesis, and the exact role of RBM3s in the regulation of miRNA expression remains somewhat unclear as well as controversial due to different findings in different studies (111, 112). RBM3 appears to promote cell cycle progression, with a modulating role in G2/M transition (113), a notion supported by the increased number of embryonic fibroblasts in the G2-phase in RBM3-deficient mice (114). In addition, RBM3 appears to have a negative regulatory function in apoptosis, through the repression of PARP-cleavage (115) and suppression of p38 signalling (116). Continuous endoplasmic reticulum (ER)-stress causes the accumulation of unfolded proteins in the ER lumen. This normally initiates an apoptotic program that RBM3 has been shown to repress (117).

In conclusion, RBM3 is a protein involved in a wide variety of functions in the normal cell. The common denominator seems largely to be that of cell salvage under harsh conditions, although much remains to be elucidated.

Function in cancer

As previously discussed, genetic mutations are underlying cause of cancer (1). The functional aspect of the mutation, and the growth advantage it conveys, depends on different scenarios. Since RBPs are involved in a multitude of processes related to posttranslational gene expression and protein synthesis, alterations due to mutations or overexpression may in turn (by alternative splicing, mislocalization or unregulated translation) affect oncoproteins and tumour suppressors (118). It has been demonstrated that RBM3 can enhance the stability and translation of the mRNA for COX-2, IL-8 and vascular endothelial growth factor (VEGF) in macrophages or cancer cells (113, 119).

RBM3 has been hypothesised to be a survival protein, helping cell survival under harsh conditions, such as hypoxia and serum withdrawal (109, 113, 120). Sureban et al found that the knockdown of RBM3 increased apoptosis in cancer cells, implying that the downregulation of RBM3 causes a mitotic catastrophe (113).

The function does however seem to be dual, aiding cancer cells to overcome the perils of the body's environment and helping them establish a tumour, while being a marker of a favourable prognosis in most major forms of cancer when overexpressed.

RBM3 has been shown to induce stemness in colorectal cancer cell lines by enhancing β-catenin signalling (121). In contrast, forced overexpression of RBM3 in prostate cancer cells was found to decrease the stemness of the cells *in vitro* (122), indicating different roles of RBM3 in different cancer forms. The results from studies investigating the role of RBM3 in solid tumours are presented in Table 2.

Table 2. Overview of studies published on different cancer types in relation to RBM3 expression

Cancer type	Proposed mechanism	Prognosis (high RBM3 expression)	Reference
Breast	-	Good	Jögi et al, 2009 (123)
Epithelial ovarian cancer	Inhibit MCM3, Chk1 and Chk2	Good	Ehlen et al 2010 (124) Ehlen et al 2011 (125)
Prostate cancer	Involve ERG and PTEN; enhance chemosensitivity; regulate CD44 splicing	Good and poor	Zeng et al 2009 (126) Zeng et al 2013 (122) Jonsson et al 2011 (127) Grupp et al 2014 (128)
Testicular	-	Good	Olofsson et al 2015 (129)
Urothelial bladder cancer	-	Good and not determined	Boman et al 2013 (130) Florianova et al 2015 (131)
Oropharyngeal	-	Not determined	Martinez et al 2007 (132)
Colorectal	Suppress GSKβ and increase β–cat signalling	Good	Venugopal et al 2016 (121) Hjelm et al 2011 (133) Melling et al 2016 (134) Wang et al 2015 (135)
Esophageal and gastric	-	Good	Jonsson et al 2014 (136)
Malignant melanoma	Inhibit MCM3	Good	Baldi et al 2003 (137) Jonsson et al 2011 (138) Nodin et al 2012 (139)
Astrocytoma	-	Unclear	Zhang et al 2013 (140)

In epithelial ovarian cancer (EOC), RBM3 was proposed to promote DNA integrity through suppression of MCM3, Chk1 and Chk2 (125). An inverse relationship between expression of RBM3 and MCM3 has also been observed in malignant melanoma (139).

High RBM3 expression has been shown to increase chemosensitivity in EOC *in vitro* (124) and low RBM3 expression was associated with high risk of treatment failure in non-seminomatous germ cell cancer (129). Furthermore, high expression of RBM3 has been demonstrated to be associated with a less favourable clinicopathological characteristics and a poorer prognosis in operated pancreatic and periampullary cancer. High RBM3 expression was however an independent predictor of an improved OS and RFS for patients that received adjuvant chemotherapy (141). In contrast to these findings, downregulation of RBM3 has been shown to increase chemosensitivity in prostate cancer *in vitro* (126) and to be related to more aggressive and earlier biochemical recurrences in a study of RBM3 expression in prostate cancer (128), although opposing results regarding biochemical recurrence have been found in another study (127).

PODXL

Function in the normal cell

Podocalyxin-like (PODXL), also known as podocalyxin, thrombomucin or Myb-ets progenitor antigen 21 (MEP21), is a transmembrane protein normally expressed in the podocytes of the kidney (142), hematopoietic progenitor cells (143, 144), vascular endothelia (145, 146) and a subset of neurons (147).

PODXL was first found as a heavily sialylated and sulphated transmembrane protein in the podocytes of the renal glomeruli (142, 148). The gene encoding the protein is located on chromosome 7q32-q33 (149) and is negatively regulated by p53 and positively regulated by Wilms tumour suppressor-1 (WT1) (150). It belongs to a family of cell surface sialomucins and is closely related to two other molecules, CD34 and endoglycan (151, 152). The protein has since its discovery been found to be essential for normal fetal development in mice, where podocalyxin-deficient individuals exhibited defects in renal development and died soon after birth due to anuria (151). The function in the glomeruli is anti-adhesive, and this feature is essential for the formation of filtrative slits in the podocytes (153). However, the effect of PODXL differs depending on the location where it is expressed. Specialized postcapillary high endothelial venules support high levels of lymphocyte extravasation are present in lymph nodes (154). In these venules PODXL acts as proadhesive ligand for L-selectin and is likely to support the tethering and rolling of lymphocytes and in this manner contributes to the recruitment of lymphocytes to secondary lymphoid tissues (152). PODXL is normally linked to the actin cytoskeleton through interactions with ezrin and the bridging protein NHERF (N+/H+ exchanger regulatory factor) proteins (155, 156) and can hence regulate cell morphology.

Function in cancer

The first description of upregulated expression of PODXL in cancer was in testicular cancer, where is was found to be a marker of nonseminomatous germ cell tumours (157). It has also been proposed to play a part in the process of

epithelial to mesenchymal transition (EMT) (158). This is the process where epithelial cells undergo a rearrangement of structure and changes in properties and acquire a mesenchymal phenotype. Normally, epithelial cells are connected to each other with E-cadherins and to the extracellular matrix by molecules such as integrines, and unable to move independently of each other. The process of EMT includes the degradation of E-cadherin and integrines to reduce interconnectivity between cells and to the extracellular matrix. Proteins responsible for the restructuring of the cytoskeleton are also produced and dynamic actin remodelling is necessary for successful EMT. Further expression of genes leads to expression of proteins and structures responsible for the invasion of the extracellular matrix and cellular motility. These changes within the cell result in the transition into a mesenchymal cell, capable of migration and invasion (158, 159). PODXL has been reported to be markedly increased during TGF-β induced EMT and the cytoskeleton assembly to be dependent on the production of PODXL (158). Expression of PODXL has been demonstrated to increase matrix metalloproteates and increase the activity of survival pathways, such as MAPK/ERK and PI3K-AKT (160).

Prior to the initiation of this thesis work, PODXL expression had not been described in UBC. It had however been described to be associated with a more aggressive tumour phenotype and adverse survival in breast cancer (161), associated with more aggressive tumours in prostate cancer (160, 162) and studies on breast and prostate cancer cell lines have reported that PODXL enhances the malignant and migratory potential in an ezrin-dependent manner (160). Furthermore, high PODXL expression had been demonstrated to be significantly and independently associated to a poor clinical outcome in renal cell carcinoma (163) and in colorectal cancer (164).

Since then, PODXL expression has been found to be associated with a more aggressive tumour phenotype and/or adverse outcome in further studies on colorectal cancer (164-167), ovarian cancer (168), glioblastoma multiforme (169), ovarian cancer (168), gastric and esophageal cancer (170) and pancreatic cancer (171).

Material and Methods

Patient cohorts

Paper I

The cohort in paper I was obtained from a register from Uppsala University Hospital, where all patients with newly diagnosed UBC have been registered since 1984 and paper I encompassed patients who were diagnosed up until Dec 31st 2005. The number of Ta tumours was however reduced, as this was the predominant group. A total number of 343 patients were included in the TMA used for the study. Clinical data and follow up regarding recurrence, progression, vital status and cause of death were obtained from the medical charts and pathology records.

Paper II

Two patient cohorts were used in paper II. The first cohort was a consecutive cohort of all patients with newly diagnosed UBC at Skåne University Hospital, Malmö, from October 1st 2002 until December 31st 2003, for whom archival tumour specimens could be retrieved. A total of 110 patients were included in this cohort. Information on clinicopathological features were obtained from the pathology records and information on vital status was collected from the Swedish Cause of Death Registry. The second cohort was the same consecutive cohort from Uppsala University Hospital that was used in paper I.

Paper III

Patients included in the Malmö Diet and Cancer Study comprised the cohort used in paper III. Until the end of follow-up on December 31st 2010, 367 cases of incident UBC were recorded, 272 of which were included in the TMA that was used in the study. Out of these, 264 tumours were located to the bladder and included into further analyses. A flowchart describing the inclusion of cases in the

TMA is shown below. Clinical data and follow up regarding recurrence, progression, vital status and cause of death was obtained from the medical charts and pathology records.

Figure 8. Flowchart outlining the study cohort from a total of 367 registered cases of incident UBC in the Malmö Diet and Cancer Study up until December 31st 2010.

Paper IV

The study comprised a consecutive cohort of patients with UBC having undergone TURB and cystectomy at Skåne University Hospital between January 1st 2011 and December 31st 2014. A total number of 151 cases were included in the TMA. A flowchart describing the cohort is shown below. Clinical data and follow up regarding recurrence, progression, vital status and cause of death were obtained from the medical charts and pathology records.
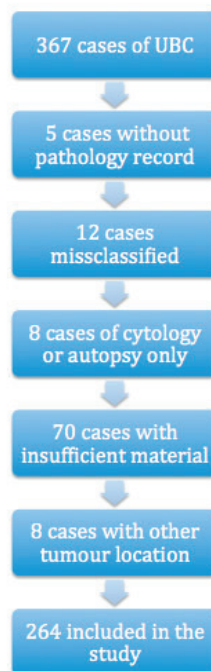
Figure 9. Flowchart outlining the study cohort from a total of 207 cases of patients having undergone surgery for UBC at Skåne University Hospital, Malmö.

Tissue microarray technique

When performing research on human cancer specimens, a finite resource, one must economize with the valuable tissue. Because of this, the tissue microarray technique (TMA) was developed, i.e. a technique where cylindrical cores (generally 0.6-3 mm) are taken from paraffin-embedded tissue and inserted into a recipient block (Figure 10). In this manner, several hundreds of tumours can be evaluated simultaneously regarding levels of DNA, RNA or proteins. A similar technique was first described as "the sausage tissue block" in 1986 (172) and has since been further developed into the TMA technique by Kononen et al (173).
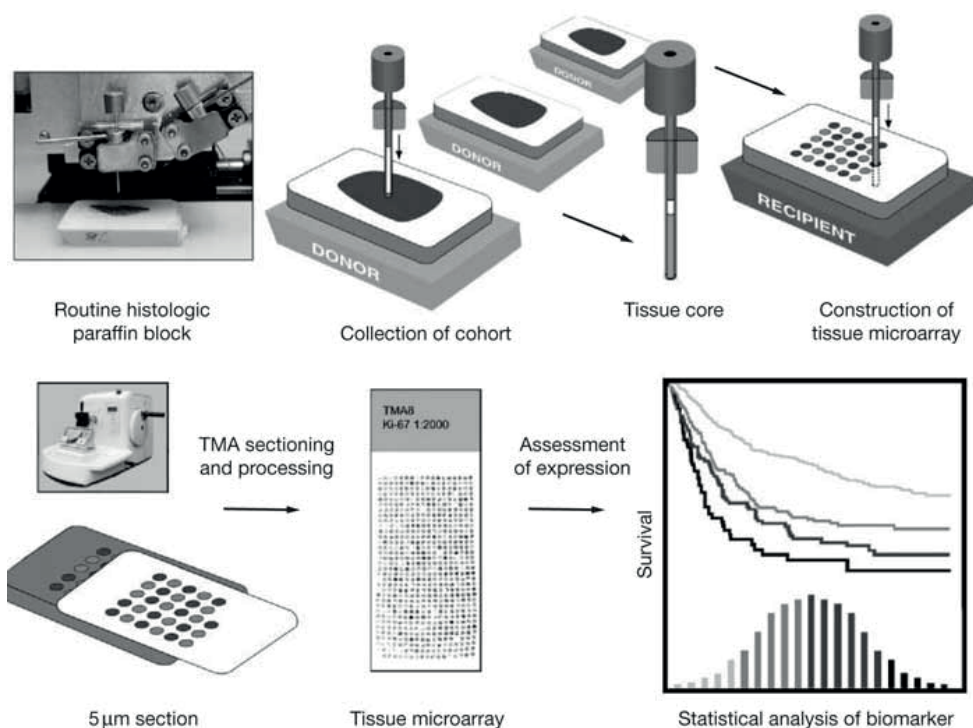
Figure 10. Schematic overview of the constructing of and utility of a TMA. Reprinted with permission from the Nature Publishing Group (174).

Tumour heterogeneity has been raised as a concern against the TMA technique. In part, this can be compensated by the use of more than one tissue core from the tumour. Studies have shown that duplicate or triplicate tissue cores are sufficient to accomplish a high level of concordance to full face sections (175, 176) and provides at least equal prognostic information as conventional tissue sections (177, 178). In bladder cancer, Nocito et al. showed that intratumour heterogeneity did not significantly affect the ability to detect clinicopathological correlations on four TMAs with a single tissue cores from each patient. This was not only true for all four TMAs combined, but also for each TMA individually (179).

Immunohistochemistry and antibodies

Immunohistochemistry (IHC) is a technique that combines immunological, anatomical and biochemical approaches to identify proteins (antigens) in a given tissue. Although known as a principle already in the 1930s, the first study

describing IHC was published in 1942 and has since evolved with e.g. better tissue fixation, antigen retrieval and labelling (180). It is based on the principle that antibodies bind to specific antigens. Antibodies can be labelled with chromogenic or fluorescent tags and, hence, visualized in a tissue. The IHC technique enables the visualization of the distribution and localization of specific cellular components both within the cells and in the context of the tissue (181). Essential for detection of the target is however the use of well-validated antibodies of high quality, as suboptimal antibodies can cause research projects to be abandoned, waste time, money and tissue samples (182).

Anti-RBM3

The antibody AMAb90655, previously named AAb030038, was used for evaluation of RBM3 expression in paper I, III and IV. It has been extensively validated in a multitude of tumours (124, 127, 129, 133, 136, 138, 139, 183).

Anti-PODXL

In paper II, cohort I was used as a test cohort for comparison of three different antibodies against PODXL: one polyclonal, monospecific rabbit antibody, HPA002110 (Atlas Antibodies, Stockholm, Sweden), validated in multiple types of human tumours (164, 170, 171, 184-187), and two monoclonal mouse antibodies; the anti-PODXL 4F10 antibody (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA), validated in ovarian cancer (188) and AMAb90667 (Atlas Antibodies AB). Validation of the antibodies HPA002110 and AMAb90667 was performed by means of epitope mapping and western blot.

Western Blot

In a paper from 1979, the authors described a method by which proteins could be separated and visualized (189). This technique is referred to as Western blot, a technique that is used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate proteins by length of the polypeptide or by the 3D structure of the protein. This method can both verify the presence of a protein as well as the relative amount present in the sample (190).
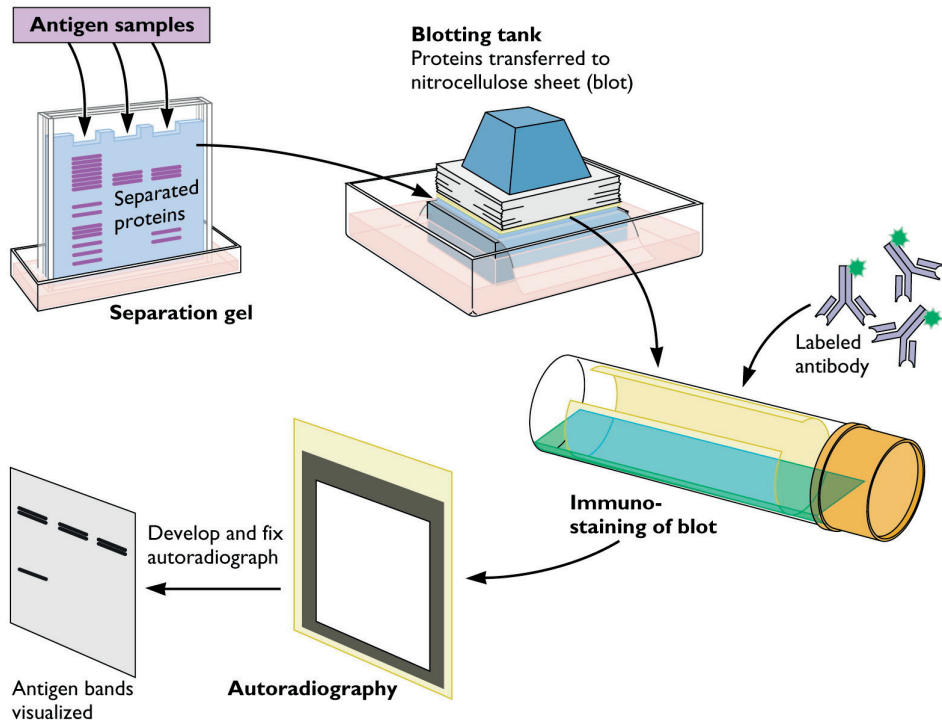
Figure 11. Schematic overview of the western blot technique. Image copyright American Society for Microbiology, Principles of virology, ASM press, Washington DC

Epitope mapping

As previously mentioned, the validation of an antibody is crucial to be of interest to researchers. Bead array assays are based on a system of coded microspheres ("beads") with a unique ID.

When validating an antibody, the antigen sequence is cut into overlapping polypeptides and coupled to a specific bead. This is achieved by linking the polypeptide with biotin that in turn is coupled with high affinity to proteins such as neutravidin that coats the beads. The beads and their matching peptides can thereafter be exposed to the investigative antibody. A secondary antibody, labelled with either a chromophore or fluorophore, then visualizes the binding of the antibody. The measurement of the fluorescence in relation to the specific bead subsequently identifies the epitope (191).

Cell lines

The two RBM3 expressing cell lines T24 and RT4 were used for evaluation of sensitivity to chemotherapy in paper IV. The T24 cell line originates from a muscle-invasive urothelial cancer in an 81-year-old female, whereas the RT4 cell line is derived from a recurrent papillary urothelial tumour in a 63 year old male (192).

Statistics

All statistical analyses were performed with SPSS version 20.0 and 23.0 (IBM, Armonk, NY, USA). Chi-square test and Spearman's correlation analyses were used to examine the associations between the investigated biomarkers and clinicopathological factors. Kaplan-Meier analysis and log rank test were used to illustrate difference in survival in strata according to the expression of RBM3 and PODXL.

To examine the potential prognostic significance of the candidate biomarkers, univariable and multivariable Cox regression analyses were applied, the latter adjusted for standard clinical risk factors in UBC. The hazard ratios (HRs) were generated with a 95% confidence interval (95% CI).

In paper I, RBM3 expression was trichotomized into negative/intermediate/high, and dichotomized into negative/intermediate versus high for analysis of progression free survival (PFS) and into negative versus positive expression for analysis of overall survival (OS).

In paper III and IV, cases were dichotomized into high versus low RBM3 expression. In paper II and III, cases were dichotomized into categories with or without membranous PODXL expression.

For evaluation of proliferation in the cell cultures in paper IV, two-way ANOVA was performed with Bonferroni post hoc test using Graphpad prism software.

Throughout, tests were two-sided and a p-value of <0.05 was considered significant.
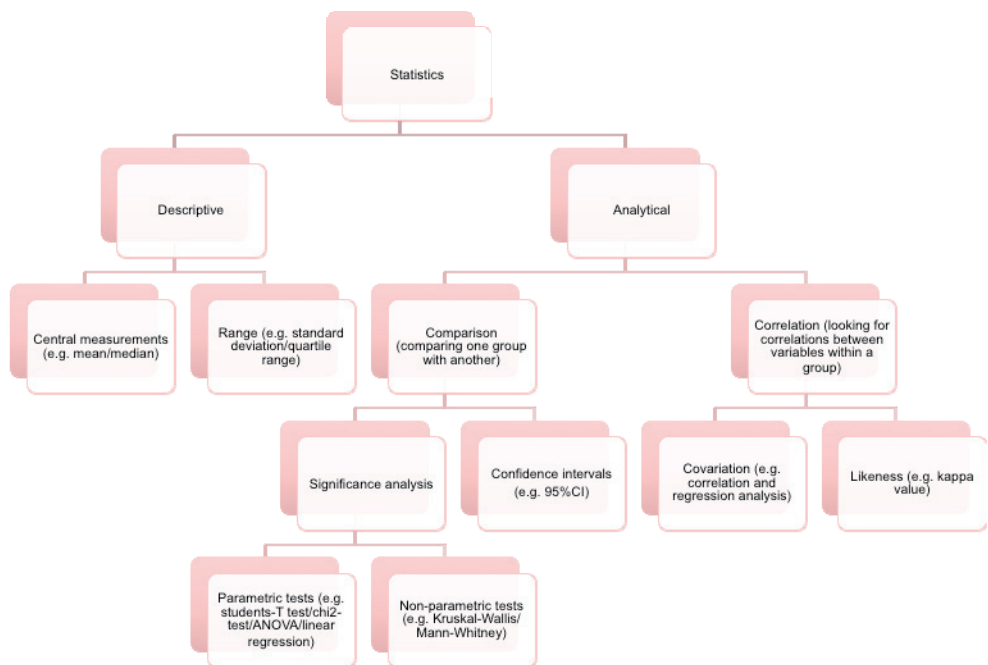
Figure 12. Simplified overview of the statistics used.

Aim of the thesis

The aim of the thesis is to evaluate the prognostic and predictive value of candidate biomarkers RBM3 and PODXL in urothelial bladder cancer.

Specific aims

Paper I: To evaluate the prognostic value of RBM3 in a cohort encompassing both NMIBC and MIBC.

Paper II: To evaluate the prognostic value of PODXL in two cohorts encompassing both NMIBC and MIBC and to further validate two of the antibodies used for detection of PODXL expression.

Paper III: To validate the prognostic value of both RBM3 and PODXL in an independent cohort encompassing both NMIBC and MIBC.

Paper IV: To evaluate the predictive value of RBM3 in relation to response to neoadjuvant chemotherapy in a cohort of patients with MIBC and to further investigate the role of RBM3 in response to chemotherapy *in vitro*.

Summary of results and discussion

Paper I

In this paper, we examined the potential prognostic value of RBM3 in a consecutive cohort of patients with UBC (n=344). RBM3 expression could be evaluated in 343 (99.7%) of the tumours. Negative RBM3 staining (NS=0) was denoted in 77 (22.4%) cases, weak-strong intensity in <75% (NS=1-6) in 213 (62.1%) cases and strong staining in 53 (15.5%) cases. Reduced RBM3 expression was significantly associated with more aggressive tumours, i.e. higher T-stage (p<0.001) and high grade disease (p=0.004) as well as higher age at diagnosis (p=0.013). RBM3 expression was not associated with the occurrence or the frequency of local recurrence.

In the full cohort, Kaplan-Meier analysis revealed a stepwise reduced DSS and shorter 5-year OS with decreasing RBM3 expression, with the shortest DSS and 5-year OS for patients lacking RBM3 expression (both p<0.001). Cox regression analysis confirmed these associations, and the prognostic value of RBM3 expression remained significant in analysis adjusted for age, sex, T-stage and grade in relation to both disease specific (unadjusted HR 2.55; 95% CI 1.68-3.86 and adjusted HR 1.65; 95% CI 1.07-2.53) and OS (unadjusted HR 2.10; 95% CI 1.56-2.82 and adjusted HR 1.54; 95% CI 1.13-2.10).

In the patients with Ta and T1 tumours, progression within 24 months according to Kaplan-Meier analysis was significantly shorter for patients with RBM3 negative tumours (p=0.030) and borderline significantly shorter for patients with intermediate tumour specific RBM3 expression. A dichotomized variable with high versus negative/intermediate RBM3 expression showed a reduced PFS for patients with negative/intermediate expression tumour-specific RBM3 expression compared to patients with high tumour-specific RBM3 expression (p=0.048). Analysis of the group of non-muscle invasive tumours showed a significantly reduced 5-year OS for patients with negative tumour-specific RBM3 expression compared to patients with any tumour-specific RBM3 expression (p=0.005). A dichotomized variable with negative versus any RBM3 expression showed a significantly shorter survival in the RBM3 negative group than the RBM3 positive group (p=0.006).

Our results thus show that reduced RBM3 expression is associated with clinically more aggressive tumours and an independent factor of poor prognosis in UBC, both in an unselected population and in the Ta/T1 subgroup and may therefore be of assistance in the guidance of therapy for NMIBC. This could, if validated in the prospective setting, mean offering cystectomy to patients with low tumour-specific RBM3 expression as is recommended for other high-risk tumours, such as e.g. tumours with unusual histology (22).

Paper II

In this paper, tumours from two cohorts were examined regarding the clinicopathological correlates and prognostic significance of PODXL expression. PODXL could be evaluated in 100/110 (90.9%) cases in cohort I and in 343/344 (99.7%) cases in cohort II, with membranous expression in 21/100 (21%) cases in cohort I and in 35/343 (10.2%) cases in cohort II. Membranous PODXL staining was strongly and significantly associated with disease with more advanced T-stage (p<0.001 in both cohorts) and high-grade tumours (p=0.013 in cohort I and p=0.002 in cohort II). In cohort I, OS was significantly shorter in the population with membranous expression of PODXL. In cohort II, OS as well as DSS was significantly lower in the population with membranous PODXL (both p<0.001).

In both univariable and multivariable analyses, the latter adjusted for age, sex, T-stage and grade, the associations between membranous PODXL expression and shorter OS and DSS remained significant.

Table 3. Extract from Table 2, paper II

	Cohort I			Cohort II					
	Risk of death within 5 years			Risk of death from disease			Risk of death within 5 years		
		Univariable	Multivariable		Univariable	Multivariable		Univariable	Multivariable
	n (Events)	HR (95% CI)	HR (95% CI)	n (Events)	HR (95% CI)	HR (95% CI)	n (Events)	HR (95% CI)	HR (95% CI)
PODXL expression									
Negative	79 (33)	1.00	1.00	269 (80)	1.00	1.00	308 (144)	1.00	1.00
Positive	21 (14)	2.25 (1.20–4.22)	2.05 (1.06–3.94)	31 (21)	4.36 (2.67–7.10)	2.70 (1.60–4.55)	35 (26)	3.10 (2.03–4.72)	2.18 (1.39–3.41)

In Ta/T1 tumours, despite a low proportion of membranous PODXL expression, the presence of PODXL was an independent predictor of disease progression within 24 months (univariable HR 6.19; 95% CI 1.42-26.98 and multivariable HR

4.60; 95% CI 1.04-20.39), and also for risk of death caused by UBC (univariable HR 8.34; 95% CI 3.21-21.65 and multivariable HR 7.16; 95% CI 2.72-18.81).

In this study, we concluded that PODXL is an independent predictor of increased risk of disease progression within 24 months and cancer-specific death. The assumption based on our observations is that membranous PODXL is a biomarker that predicts a more aggressive tumour phenotype that is more likely to metastasize. This may be attributable to previous observations in other cancer forms, namely that PODXL conveys a phenotype with i) decreased cell adhesion, ii) facilitating hematogenous spread of tumour cells, iii) alteration of the cell surface and iv) increased invasion and migration, due to interactions with ezrin.

Paper III

In this cohort of incident cases of UBC in the MDCS, 262/272 (96.3%) cases could be evaluated regarding PODXL staining. Membranous staining was denoted in 27 (10.0%) cases. RBM3 expression could be evaluated in 259/272 (95.2%) cases. Negative RBM3 expression was denoted in 26 (10.0%) of cases, intermediate expression in 78 (30.1%) and high expression in 155 (59.8%) cases.

Membranous expression of PODXL was significantly associated with higher T-stage and high-grade tumours. High RBM3 expression was significantly associated with lower T-stage and low-grade tumours (p<0.001 for all). None of the investigated biomarkers were significantly associated with age at diagnosis or sex.

Kaplan-Meier analysis of the relationship between PODXL expression and 5-year OS in both the full cohort and in T1 disease showed a shorter survival for patients with membranous expression (p<0.001 for both). These associations were confirmed in univariable Cox regression analysis in the full cohort (HR 3.28, 95% CI 1.89-5.69) and in T1 disease (HR2.83, 95% CI 1.04-7.72), but did not remain significant in multivariable analysis. No association between PODXL expression and survival was seen in T2-T4 disease.

Kaplan-Meier analysis of the relationship between RBM3 expression (low/intermediate/high) and 5-year OS showed a stepwise reduced survival with decreasing levels of expression. A dichotomized variable was constructed, whereby low RBM3 expression was found to be significantly associated with shorter 5-year OS in the full cohort (p<0.001) and in T1 tumours (p=0.023). Cox regression analysis confirmed the significant association between low RBM3 expression and a shorter 5-year OS in univariable analysis in the entire cohort (HR 3.19 95% CI 2.02-5.02) and T1 tumours (HR 2.64 95% CI 1.11-6.27). In

multivariable analyses, adjusted for age, sex, T-stage and grade, RBM3 remained an independent prognostic factor in the entire cohort (HR 1.85 95% CI 1.11-3.09) and in T1 tumours (HR 2.63 95% CI 1.01-6.84). In T2-T4 tumours, high RBM3 expression was borderline significantly associated with a prolonged OS, also in multivariable analysis.

Non-classic tumour types were significantly correlated with more advanced T-stage, high grade disease, membranous PODXL and low RBM3 expression (all p<0.001).

The results led to the conclusion that this study validated the prognostic value of PODXL and RBM3, with RBM3 potentially being the most clinically useful biomarker for patients with T1 disease.

Paper IV

The prognostic and predictive significance of RBM3 expression was evaluated in a cohort of patients having undergone cystectomy due to MIBC (n=151), out of whom 69 (45.7%) had received and 82 (54.3%) had not received treatment with NAC. As many as 129 (85.4%) of patients did not have a previous history of NMIBC.

Treated patient had a significantly lower median age (64.7 vs. 73.8 years, p<0.001) but no other significant differences were seen preoperatively between the two groups. Postoperatively, the treated group had a higher proportion of pT0 disease (*p<0.001*), less CIS in the cystectomy specimen (*p=0.011*) and were node-negative (N0) more frequently (*p=0.005*). DSS was significantly longer in the treated versus in the untreated group

RBM3 expression was evaluated in TURB and cystectomy samples by IHC. A total number of 147 (94.7%) TURB cases could be evaluated regarding RBM3 expression and the corresponding number was 92 (60.9%) in the cystectomy specimens. Female sex was more common in the group with high RBM3 expression (p=0.031), but no other significant differences where found between the two groups. Survival was evaluated in strata of high (NS 6-9) and low (NS 0-4) RBM3 expression. Higher age, more advanced cT and pT-stage and no NAC were significant predictors of an unfavourable prognosis, though only clinical T3-stage remained an independent prognostic factor in the adjusted analysis. RBM3 expression was not prognostic.

Next, a combined variable with four different categories; low RBM3 expression without NAC, high RBM3 expression without NAC, low RBM3 expression with NAC and high RBM3 expression with NAC, was analysed in relation to RFS and

DFS. There was a significantly reduced RFS in cases with high RBM3 expression who were not treated with NAC compared to those with high expression who received chemotherapy. DFS did not differ between the groups.

The *in vitro* studies showed that silencing RBM3 rendered T24 cells less sensitive to the effects of cisplatin and gemcitabine, and that cells after silencing, as expected, had lower levels of RBM3 mRNA, but also lower levels of IL-8 and COX-2 mRNA. RT4 cells had less RBM3 expression from the start and exhibited an innate resistance to both tested drugs.

From these results we concluded that RBM3 expression in UBC appears to predict response to chemotherapy, with the best prognosis in the group with high RBM3 expression treated with NAC and the worst for those with high RBM3 expression who did not receive treatment. These findings were further corroborated with *in vitro* experiments. This is potentially due to the interactions of RBM3s with COX2 and IL8, with angiogenic effects downstream, and speculatively due to the increased fraction of cells replicating their DNA because of the proliferative effects of RBM3, thus enabling gemcitabine and cisplatin to exert their effects. The ability to predict response to chemotherapy could help select patients for therapy, and ameliorate the therapy of those that stand to gain from therapy, as the side-effects of chemotherapy may be easier to accept for both patients and oncologists when the therapy causing them is likely to induce tumour response. An important aspect of predicting response is also to spare patients who are not likely to benefit from therapy from the side effects of chemotherapy.

Strengths and limitations

This thesis summarizes findings from almost 900 individual patients, from 4 independent cohorts. At least two observers examined the same tumours independently and blinded to clinical data. The antibodies used were throughout well validated, and three different antibodies examined in paper II were 100% concordant regarding the detection of PODXL in paper III. *In vitro* data supported the value of RBM3 as a predictor of response to chemotherapy in paper IV.

However, some weaknesses can also be identified:

The immunohistochemical technique has both strengths and weaknesses, as has been touched upon previously. While this approach limits the number of markers that can be investigated simultaneously and requires well-validated antibodies, IHC is a reliable method for detection and quantification of proteins in a subcellular context.

The TMA technique has its pros and cons. Some of the critique that has been raised against this method is that tumour heterogeneity may not be reflected in small tissue cores. To minimize this potential pitfall, duplicate cores were sampled in paper I-III and in triplicate cores in paper IV. A potential underestimation of the investigative biomarkers can however not be ruled out.

Another potential "blind spot" is that data on concomitant CIS and treatment data were not available for the patients in paper I-III, nor was performance status included in any of the cohorts.

The studies were performed on retrospective patient cohorts, although cohort I was prospectively collected and cohort III encompassed all patients with UBC occurring in the prospective Malmö Diet and Cancer Study. With retrospective studies there is always a risk of selection bias and confounding factors.

Conclusions

Low RBM3 expression and membranous expression of PODXL in UBC is associated with adverse clinicopathological factors, i.e. higher T-stage and high-grade disease.

Low RBM3 expression independently predicts a poor prognosis in UBC, with the largest potential prognostic value in NMIBC.

High RBM3 expression is associated with response to chemotherapy, both *in vitro* and *in vivo*.

Membranous expression of PODXL is associated with adverse clinicopathological factors, i.e. higher T-stage and high-grade disease.

Membranous expression of PODXL is associated with poor prognosis in UBC.

Future perspectives

A dilemma with NMIBC tumours is that a considerable proportion of patients are either being over- or undertreated. Although only a minority of patients with T1 tumours progress into MIBC, these patients appear to have a worse prognosis than those with primary MIBC (193).

Because of a high median age at diagnosis, with a peak incidence in the seventh decade of life, and a strong link to tobacco smoking, patients with advanced UBC often have significant comorbidities. This fact, paired with the absence of treatment predictive markers, puts patients with advanced UBC at risk of receiving treatment that causes them more harm than good, either delaying or precluding them from curative surgery or, in the metastatic setting, by impairing their quality of life without prolonging their survival.

Although the findings in this thesis need to be validated in a prospective setting, there are strong indications that both PODXL and RBM3 have potential as prognostic biomarkers in UBC. If these findings are validated, the biomarkers could have implications in the precystectomy setting, for the treatment stratification of NMIBC. Apart from performing a prospective validation of the prognostic value of RBM3 and PODXL, the examination of differentially expressed genes after the silencing of their respective genes would be of interest to gain further insight into their roles in UBC.

Another highly interesting topic for further study is that of the treatment predictive value of RBM3. The increased sensitivity of tumour cells with high expression of RBM3 to chemotherapy, including cisplatin and gemcitabine, demonstrated in paper IV could have treatment implications for patients with UBC that may benefit from NAC. A randomized, controlled trial of patient with high tumour specific expression of RBM3 into treatment arms with either cisplatin or gemcitabine based treatment could elucidate the potential predictive value of RBM3. If combination treatment with gemcitabine in these patients was proven equal or superior to MVAC, a treatment regime generally considered to be more tolerable could become the weapon of choice for this population.

In addition, considering that many patients with MIBC have comorbidities that disqualify them from NAC, a trial randomizing patients with tumours with high RBM3 expression with poor performance status (PS) and/or pre-existing

conditions that disqualify them from combination treatment into either cystectomy up front or to the treatment with single agent gemcitabine as neoadjuvant treatment could also be of interest. As patients with poor PS and comorbidities also have limited room for treatment in the metastatic setting, a potential survival benefit in these patients could prove very useful.

Populärvetenskaplig sammanfattning

I år får drygt 2500 svenskar cancer i urinblåsan. Det första tecknet på sjukdomen brukar vara blod i urinen, något som får de flesta att söka vård. I Sverige upptäcks 70-80% av fallen tidigt, då sjukdomen enbart finns i blåsans ytliga skikt, men sjukdomsförloppet för dessa patienter är svårt att förutsäga och återfall i sjukdomen är vanliga. Vi vet dock inte på förhand vilka som drabbas. Resterande 20-30% har en mer avancerad sjukdom när den upptäcks, och sjukdomen har då spridit sig till blåsans muskellager och/eller vidare. För dessa patienter, de med så kallade muskelinvasiva tumörer, krävs kirurgi för att ha chans att uppnå bot. För att öka sannolikheten för detta får många patienter cellgiftsbehandling innan de opereras, så kallad neoadjuvant cytostatika, för att få bort cancerceller som senare kan ge upphov till dottertumörer. Man har i stora internationella studier sett en överlevnadsvinst för dem som får sådan behandling. Det är en tuff behandling, där vi i vissa fall orsakar väldigt allvarliga biverkningar som i värsta fall fördröjer eller omöjliggör botande kirurgi. Vi vet inte på förhand vilka patienter som kan dra nytta av cytostatikan. Sedan sjukdomen spritt sig i kroppen är bot oftast utom räckhåll.

RBM3 och PODXL är proteiner som i den friska kroppen har viktiga uppgifter, både under fosterutveckling och hos fullvuxna. Ökat uttryck av båda dessa proteiner ses även i en del cancertumörer. När RBM3 uttrycks annorlunda i cancer har det i många tumörformer varit sammankopplat med god prognos. PODXL har däremot varit kopplat till dålig prognos, särskilt då det finns på cancercellernas yta.

I de tre första arbetena i denna avhandling såg vi att lågt uttryck av RBM3 och högt uttryck av PODXL var sammankopplat med "elakare" tumörer och kortare överlevnad. Detta gällde både hela patientgruppen och patienter med ytliga tumörer. Att bättre kunna förutspå dessa patienters risk att dö i sjukdomen skulle kunna göra stor nytta eftersom vi då kan identifiera dem som behöver mer aggressiv behandling redan i ett tidigt skede.

I det fjärde delarbetet undersökte vi alla patienter som genomgått kirurgi med avlägsnande av urinblåsan i Skåne mellan åren 2011 och 2014. Knappt hälften av patienterna hade fått cellgifter inför operationen, detta för att minska risken att tumören skulle ge upphov till metastaser (dottertumörer). Vi såg där att högt uttryck av RBM3 i tumörceller verkade kunna förutspå om en patient skulle kunna

dra nytta av cellgiftsbehandlingen. De patienter som hade högt uttryck av RBM3 som inte fick cellgifter hade kortast överlevnad, medan de med högt uttryck av RBM3 som fick cellgifter levde längst. I gruppen som hade låga nivåer av RBM3 i tumörcellerna skilde sig inte överlevnaden mellan behandlade och obehandlade patienter. Detta tyder på att nivåer av RBM3 i tumören kan förutspå dels vilka patienter som kan dra nytta av cellgiftsbehandling och dels de som har mindre chans att vara behjälpta av den. För att bekräfta sambandet mellan RBM3 och effekt av cytostatikabehandling gjorde vi även experiment på cancerceller som styrkte det vi såg hos patienterna, nämligen att de celler som hade högre nivåer av RBM3 växte sämre än de som hade låga nivåer av RBM3 då vi utsatte dem för cellgifter.

De fynden som gjorts under detta avhandlingsarbete är viktiga, men innan RBM3 eller PODXL kan börja användas i kliniskt bruk krävs det ytterligare studier, framför allt så kallade prospektiva (framåtblickande) studier. Om vi lyckas bekräfta våra fynd innebär det att vi har tillgång till verktyg som gör att vi kan skräddarsy behandling till patienter med cancer i urinblåsan. Detta gäller såväl för dem som har tidig sjukdom, där en bra riskbedömning är den viktigaste faktorn, som för dem med muskelinvasiv sjukdom, där bedömning av vem som ska få cellgiftsbehandling är det viktigaste. Det skulle vara något väldigt värdefullt, både för patienter och för läkare.

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