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Molecular Characterization of Bladder Cancer Subtypes

Pontus Eriksson

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Abstract

Bladder cancer is one of the most common malignancies world-wide, and in Sweden approximately 3000 cases are diagnosed annually. Even though bladder cancer is so common there is low public awareness of the disease, and it has historically been less studied compared to other common types of cancer. This is reflected in the fact that both the treatment methods and outcome for patients have remained relatively unchanged during the last 30 years.

During the last 5 years our knowledge of the disease has increased greatly through extensive molecular profiling. We have also witnessed the rise of immunotherapy treatments which holds great promise for bladder cancer treatment.

Bladder cancer has now been studied on the transcriptomic, genomic, epigenomic, and proteomic level and the results are on the verge of clinical translation. Detection, risk stratification, and treatment prediction are just a few of the key areas where the molecular research can help us improve the management of bladder cancer.

This thesis provides a broad overview of both clinical and molecular aspects of bladder cancer.
The overarching aim of the included research papers has been to further improve our understanding of the molecular biology of the disease.

Key words
Bladder Cancer, Urothelial Carcinoma, Molecular Subtypes, Genomic Characterization, Gene Expression

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Molecular Characterization of Bladder Cancer Subtypes

Pontus Eriksson

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List of papers

The thesis is based on the following original publications:

I. **Eriksson P**, Aine M, Sjödahl G, Staaf J, Lindgren D, Höglund M. Detailed Analysis of Focal Chromosome Arm 1q and 6p Amplifications in Urothelial Carcinoma Reveals Complex Genomic Events on 1q, and SOX4 as a Possible Auxiliary Target on 6p.
PLoS One 2013;8:e67222.

II. **Eriksson P**, Aine M, Veerla S, Liedberg F, Sjödahl G, Höglund M. Molecular subtypes of urothelial carcinoma are defined by specific gene regulatory systems.
BMC Med Genomics 2015;8:25.

III. **Eriksson P**, Sjödahl G, Chebil G, Liedberg F, Höglund M.
HER2 and EGFR amplification and expression in urothelial carcinoma occurs in distinct biological and molecular contexts.
Oncotarget 2017;8:48905-14.

IV. Sjödahl G, **Eriksson P**, Liedberg F, Höglund M.
Molecular classification of urothelial carcinoma: global mRNA classification versus tumour-cell phenotype classification.
J Pathol 2017;242:113-25.

V. Marzouka NA, **Eriksson P**, Rovira C, Liedberg F, Sjödahl G, Höglund M.
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1. Sjödahl G, Lövgren K, Lauss M, Patschan O, Gudjonsson S, Chebil G, Aine M, **Eriksson P**, Månsson W, Lindgren D, Fernö M, Liedberg F, Höglund M.
   Toward a molecular pathologic classification of urothelial carcinoma. Am J Pathol 2013;183:681-91.

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   TGFbeta attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. Nature 2018;554:544-8.

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Re: Thomas Powles, Robert A. Huddart, Tony Elliott, et al. Phase III, Double-blind, Randomized Trial that Compared Maintenance Lapatinib versus Placebo after First-line Chemotherapy in Patients with Human Epidermal Growth Factor Receptor 1/2-positive Metastatic Bladder Cancer. J Clin Oncol 2017;35:48-55: Knowing HER2 Status is Not Enough: A Molecular Subtype Approach to Bladder Cancer is Also Needed. Eur Urol 2017;72:e135-e6.

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Abbreviations

BC	Bladder cancer
BCG	Bacillus Calmette-Guerin
CGH	Comparative genome hybridization
CSS	Cancer specific survival
CUETO	Club Urologico Español de Tratamiento Oncologico
DDR	DNA damage and repair
EORTC	European Organization for Research and Treatment of Cancer
IHC	Immunohistochemistry
ISUP	International Society of Urological Pathology
LOH	Loss of heterozygosity
MI	Muscle invasive
MIBC	Muscle invasive bladder cancer
NMI	Non-muscle invasive
NMIBC	Non-muscle invasive bladder cancer
OS	Overall survival
PFS	Progression-free survival
RFS	Recurrence-free survival
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
TCGA	The Cancer Genome Atlas
TF	Transcription factor
TMA	Tissue microarray
TURBT	Transurethral resection of the bladder tumor
UC	Urothelial carcinoma
UPKs	Uroplakins
WHO	World Health Organization

1. Introduction

1.1. The function and biology of the bladder

The urinary bladder is a muscular sac composed of several distinct tissue layers, and functions as a temporary reservoir for urine produced by the kidneys. A primary function of the kidneys is to remove various ions, solutes, and metabolic waste compounds, some potentially toxic, from the blood stream. As such the bladder wall is continuously exposed to high concentrates of compounds in the urine. It is crucial that the bladder prevent any exchange of waste products and water between urine and the tissue and blood throughout the micturition cycle. The permeability barrier is achieved by the urothelium, the innermost mucosal lining of 5-7 cell layers of stratified epithelium which coverers most of the urinary tract, including the renal calyces and pelvises, the ureters and the bladder, and to some extent also the urethra. It is composed of basal, intermediate, and luminal cells, where the exceptionally tight barrier is achieved primarily by the unique specialization of the apical plasma membrane of differentiated superficial luminal cells (also known as umbrella cells). The membranes of these cells contain urothelial plaques (also known as asymmetric unit membrane (AUM) plaques), which are composed of highly ordered hexagonally arranged 2D crystals of transmembrane uroplakin proteins [1]. Together with high resistance tight junctions and an apical glycan layer, these properties results in an exceptional barrier to water, solutes in the urine, as well as toxic compounds. This cell layer is both mechanically resilient and compliant which allow for filling and voiding of the bladder. Beneath the umbrella cells there resides a variable number of intermediate cells, and a single layer of basal cells in adhesive contact with the basal lamina. Renewal of the urothelium occurs by asymmetrical division of progenitor cells located in the basal layer. The proliferation rate of the urothelium is normally very low, but can rapidly increase in response to injury or inflammation. Past the basement membrane, the bladder wall is composed of stroma (also called submucosa or lamina propria), an elastic layer of connective tissue rich with blood vessels and nervous tissue. This in turn is surrounded by smooth muscle (also called muscularis propria or detrusor muscle), that on the outside is covered by a layer of perivesical fat (Figure 1).

1.2. Urothelial carcinoma

Bladder cancer (BC) is one of the most common malignancies, with approximately 3 000 annual cases diagnosed in Sweden [2-4], with 430 000 cases and 165 000 deaths recorded worldwide in 2012 [5]. In Sweden it ranks as seventh most common cancer overall and as the fourth most common in men due to a large gender disparity, where the incidence is roughly 3-4 fold higher in men compared to women [2-4]. There is also a large geographic variation in incidence [6,7]. Bladder cancer risk is associated with old age, with a median age of 73 at the time of diagnosis in Sweden [2]. The disease can occur at any age, but is uncommon below the age of 50 [2]. The vast majority of bladder cancers originates from the urothelial cell layer, and is thus defined as urothelial carcinoma (UC). For this reason, I will use the terms "urothelial carcinoma" and "bladder cancer" somewhat interchangeably in this thesis, but note that bladder cancers can have other cellular origins.

1.3. Epidemiology and etiology of bladder cancer

The leading risk factor for bladder cancer is tobacco use, with cigarette smokers showing an approximately threefold higher risk compared to non-smokers [8]. It is estimated that roughly one fourth of bladder cancers in women and close to half of all bladder cancer in males can be attributed to smoking [8]. Smoking is expected to remain the leading preventable cause of bladder cancer. It is estimated that roughly one fifth of males in North America, India, and Europe smokes, while more than half of adult men in China smokes [9-13]. While many countries have seen a decrease in the proportion who smokes, global population growth have increased the total number of daily smokers from 721 million in 1980 to 967 million in 2012 [14]. As early as 1895 it was noted that occupational exposure to carcinogens was linked with bladder cancer [15]. Modern meta-analyses show that occupational factors constitute the second most important risk factor for bladder cancer, linked to 5% of all BC (7% for BC in males) [8,16] . In particular, workers that are exposed to carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons, combustion products, or heavy metals have an increased risk of developing bladder cancer. Risk groups include tobacco workers, dye, rubber, metal, or petroleum workers, chimney sweeps, and hairdressers. However, in many countries the occupation-associated risk has decreased through health and safety regulations [16]. The gender disparity remains poorly understood, however it is likely that biological factors such as anatomy and sex hormone composition plays a role, as gender-specific risk differences persists even after adjusting for major risk factors such as smoking [17,18].

2. Histopathology of bladder cancer

2.1. Diagnosis and staging

Urothelial carcinoma presents mainly as a papillary growth into the bladder lumen, a flat lesion, or as a solid tumor growing into the bladder wall. Most bladder cancers (64%) are diagnosed after a patient presents with macroscopic blood in the urine (hematuria) [19]. Macroscopic hematuria is the isolated symptom with highest positive predictive value for cancer, with a malignancy detection rate of 5−34 % depending on the composition of the investigated population [20-22]. Lower risk symptoms include dysuria, bladder pain, frequent urination or sudden urge, and frequent urinary tract infections. A patient that present with macroscopic hematuria or is suspected of having bladder cancer undergoes an examination of the bladder wall with an endoscope under local anesthesia (cystoscopy). The examination frequently also includes urine testing and cytology to detect infections and atypical cells, respectively. Radiological imaging of the bladder and kidneys (CT-urography) is used to rule out causes for hematuria in the upper urinary tract and for staging if a bladder cancer is diagnosed. If a tumor lesion is found in the bladder the first step of the initial management consists of a transurethral resection of the bladder tumor (TURBT), where a resectoscope is used to remove the lesion, or to take a biopsy in case of a muscle invasive tumor when radical cystectomy is anticipated. Thus, the TURBT is both a therapeutic and a diagnostic procedure, where successful removal of the entire lesion may be curative if the tumor is not invasive. An initial assessment of the clinical tumor stage (cT) can be made based on the results of palpation, cystoscopy, and imaging tests; however most important is the pathological stage (pT). The pathological evaluation is performed on the resected TURBT sample to determine the depth of invasion. Bladder cancer is categorized based on invasion and spread, using the Tumor, Node, Metastasis (TNM) classification system (Table 1)[23]. The tumor stage describes how deep the tumor has grown into the bladder wall, and whether it has extended to other organs. Bladder cancer is largely divided into non-muscle invasive bladder cancer (NMIBC) of stages Ta, Tis, and T1, and muscle invasive bladder cancer (MIBC) of stages T2-T4 if the tumor growth extends into the muscle layer or beyond.

Stage Ta tumors are non-invasive superficial tumors that usually show a papillary growth into the bladder lumen. They are strictly confined to the epithelial mucosa and do not invade through the basal membrane. If the carcinoma is devoid of a papillary morphology and instead displays a flat growth pattern it is classified as Tis (carcinoma in situ). Tumors of stage T1 have invaded the subepithelial stromal layer, but not the muscle layer. If a tumor invades the muscle layer of the bladder it is of stage T2 or higher, depending on the extent of invasiveness. T2 tumors extends into to the muscle layer, whereas T3 tumors show perivesical spread, and T4 tumors show outgrowth to surrounding tissues such as the prostate, uterus, vagina, or the pelvic or abdominal wall. Additional sub classification of the T2-T4 stages reflects the extent of invasion. The stage designations TX indicate that the primary tumor could not be assessed, while T0 indicates that there was no evidence of a primary tumor. The node status and metastasis status is critical for the prognosis of the patient, but more challenging to determine. Imaging methods such as CT or PET/CT can detect enlarged pelvic lymph nodes, but pathological examination of post-surgery tissue is usually required to confirm if the tumor has spread to the lymph nodes, due to the low sensitivity of imaging tests [24,25]. Node status is categorized as N0-N3 based on the location and number of positive lymph nodes, while M0-M1 status describes the presence of distant metastasis. Nx and Mx indicate that node and metastasis status could not be assessed.

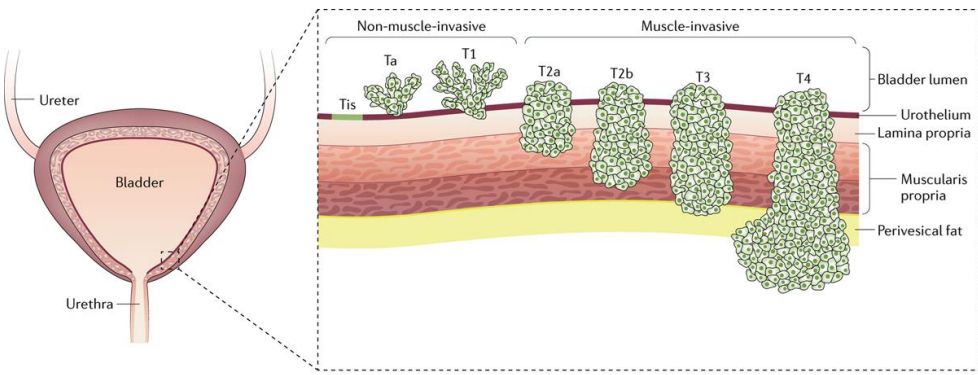


Figure 1. Illustration of the bladder composition and tumor invasion by stage. Adapted from Sanli et al. (2017) [26] , with permission from Springer Nature.

Table 1. Tumor, Node, Metastasis (TNM) classification system for bladder cancer. [23]

T – Primary Tumor	
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: 'flat tumor'
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue:
T3a	microscopically
T3b	macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostate stroma, seminal vesicles, uterus or vagina
T4b	Tumor invades pelvic wall or abdominal wall
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor

N – Regional Lymph Nodes	
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N2	Metastasis in multiple regional lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3	Metastasis in a common iliac lymph node(s)
NX	Regional lymph nodes cannot be assessed
M – Distant Metastasis	
M0	No distant metastasis
M1a	Non regional lymph nodes
M1b	Other distant metastasis

Approximately 70%-80% of newly diagnosed bladder cancers are NMI, while the remaining 20%-30% are muscle invasive. Among diagnosed NMIBC about 50-70% presents as stage Ta, 20-40% as T1, and 5-10% with Tis/CIS [27-29]. Although the prognosis for patients with NMIBC is generally good, they have a high propensity for tumor recurrence and a risk for progression to a higher tumor stage. More than half of all patients with NMI bladder cancer recur with a later tumor at some point, with 5%-20% progressing to muscle invasive disease [28-31]. Patients with stage T1 bladder cancer pose a unique challenge due to the heterogeneity of clinical outcome for this group. Because stage T1 tumors have not yet invaded the muscle, but still display aggressive properties, it is difficult to determine if they should be treated like a non-invasive tumor, or whether they should be treated like a muscle invasive tumor which results in significantly more treatment-related adverse effects for the patient. Roughly half of patients with T1 staged disease will be cured by clinical intervention, however one third of aggressive T1 tumors progress to muscle invasive disease and the cancer specific mortality is around 15-20% [32-35]. To improve the pathological risk assessment, several substaging methods have been proposed and tested for T1 tumors [36]. Some anatomy-based methods, such as "T1 a/b" and "T1 a/b/c" substaging, utilize the depth of invasions in relation to the muscularis mucosae [37-40], while some systems, e.g. "T1 m/e", are based on the depth of invasion measured in millimeters [41]. These methods of staging have been reported to have predictive value for progression-free survival (PFS) and recurrence-free survival (RFS), but because of inconsistent results in terms of predictive value and the difficulty of consistently and accurately determining the actual depth of invasion in the TURBT tissue, these systems have not been widely adopted.

Muscle invasive bladder cancer is an aggressive disease, which rapidly metastasizes and is lethal within years if untreated [42]. With current treatment regimes, Stein et al. (2001) found the 5 year recurrence-free survival for patients with organ confined disease without node metastasis to be 89% for T2 and 78% for T3a staged tumors, decreasing to 50% and 41% respectively if node metastasis were present [43]. For patients with non-organ confined disease (stage T3b and T4) and no node involvement the 5 year RFS was 58%, decreasing to 30% if node positive [43]. More recent results from a study of nearly 15 000 patients were similar, indicating a 5 year cancer specific survival of approximately 85% for patients with T2 node negative disease, 50% in T2 node positive, 60% in T3/T4 node negative, and 30% in patients with T3/T4 node positive disease [44].

2.2. Tumor grading

The tumor is also given a histologic grade based on the degree of cellular atypia, growth pattern, and mitotic activity. The heterogeneous behavior of non-muscle invasive bladder tumors is main reason for the importance of grading in clinical decision-making. While the tumor stage is the most important factor for treatment selection, the grade of the tumor reflects the inherent aggressiveness of the tumor [28,29]. The tumor grade serves as an important prognostic indicator particularly in non-muscle invasive bladder cancer, where a high histological grade indicate that a particular tumor has a higher risk of recurring or progressing to muscle invasive disease [29], and that a more aggressive treatment and monitoring should be considered. Urothelial carcinomas lend themselves better for histological grading than many other malignancies since both the growth architecture and cytological features are readily analyzed in the TURBT specimen. Unfortunately, three parallel systems are used for grading tumors; the "WHO 1973" and the subsequent "WHO 1999" update, and the "WHO/ISUP 2004/2016" systems. The WHO 1973 grading system was published in 1973 by the World Health Organization and uses a three tiered system (Grade 1-3) [45] where increasing grade reflect the degree of cellular abnormality. The WHO 1973 grading system is criticized for ambiguous and poorly defined separation criteria between grade 1 and 2, and between grade 2 and 3, respectively. The effect was that only very low risk cases were classified as grade 1 and only those with extreme cellular abnormality as grade 3, with the majority considered grade 2 [46]. This resulted in confusion on how to treat the large group of grade 2 tumors that were shown to include high risk patient with up to 20% progression rates and 13-20% cancer specific death [47,48]. The WHO 1973 grading system was refined in the WHO 1999 update, which provided clearer distinctions between the different grades and included the category "papillary urothelial neoplasms of low malignant potential" (PUNLMP) reserved for small tumors that resemble exophytic urothelial papilloma, with increased cellular proliferation and low cellular atypia, many of which were classified as grade 1 in the 1973 system [49]. The aim of the low risk PUNLMP category, which encompasses neoplasms that are neither benign nor malignant, was partly to avoid labeling the patients with the term "cancer" in order to decrease the associated psychosocial effects. This category was controversial and subsequent studies have reported a recurrence and progression rate of approximately 10-20% and 10%, respectively, suggesting they should be monitored similar to stage Ta tumors [50-54].

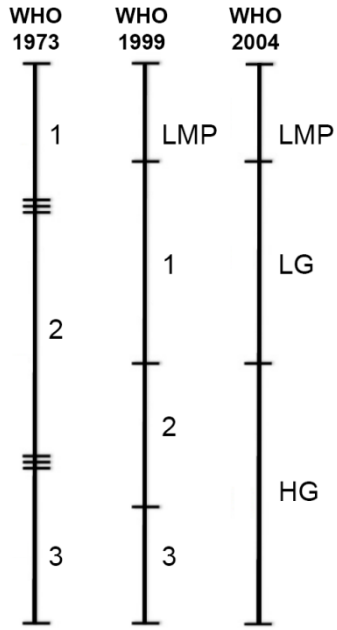
The most frequently used grading system is the WHO/ISUP 2004/2016 (International Society of Urological Pathology) grading system first proposed in 1998, and updated in 2004 and 2016. The WHO/ISUP system introduced the PUNLMP category also used in the WHO 1999 system, but differs from the 1973 and 1999 systems by separating tumors into only two categories; low grade (LG) and high grade (HG), with "high grade" largely equating to those of grade 2 and 3 in the WHO 1999 system (Table 2). The two tiered grading system was proposed to reduce the inter-observer variability that was seen in the WHO 1973 system, and to address the poor definitions of the 1973 system, but sparked an extensive, still ongoing, scientific debate regarding the value of the different grading systems [53,55-61]. Proponents of the three tiered system have argued that the issues of the 1973 system were addressed in the WHO 1999 update and that the distinction between grade 2 and grade 3 is of both prognostic and biological significance [46,61].

Multiple studies have compared the prognostic value and reproducibility of the different grading systems. Most studies compare the WHO 1973 against the WHO/ISUP 2004/2016 system, but often without comparisons to the WHO 1999 system as it is the least commonly used system. A comprehensive meta-analysis comparing the 1973 and 2004/2016 system in non-muscle invasive bladder cancer found an overall recurrence rate of 33%, 44%, and 65% for G1 vs G2 vs G3, respectively, with 28%, 43%, and 58% for PUNLMP, LG, and HG tumors respectively [46]. Progression rates were 3%, 9%, and 28% for grades 1-3, with 2%, 4%, and 19% for PUNLMP, LG, and HG. The interobserver reproducibility remained an issue with both systems, with "poor" reproducibility using WHO 1973 (kappa 0.003-0.356, G1 vs G2 vs G3), and "poor to fair" for the WHO 2004/2016 system (kappa 0.17-0.516, PUNLMP vs LG vs HG). It must be noted that in stage T1 tumors where risk assessment may be most critical, the WHO 2004/2016 essentially becomes a one-tiered grading system as most tumors will be classified as high grade, while the WHO 1973 and WHO 1999 systems classifies T1 tumors into either grade 2 or grade 3 with significant differences in survival measurements [62].

In Sweden it is recommended that both the WHO 1999 and the WHO/ISUP 2004/2016 grades are reported. A 2011 survey of western European pathology laboratories reported that 51% used the WHO/ISUP 2004/2016 system, 43% used the WHO 1973 system, and 31% used the WHO 1999 system, and many reported both WHO 1973 and WHO/ISUP 2004/2016 grades [63].

Table 2. Pathological grading systems of bladder cancer and their approximate interrelationship.

WHO 1973 grading
Urothelial papilloma
Grade 1: well differentiated
Grade 2: moderately differentiated
Grade 3: poorly differentiated
WHO 1999 grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade urothelial carcinoma, Grade 1
High-grade urothelial carcinoma, Grade 2
High-grade urothelial carcinoma, Grade 3
WHO 2004/2016 grading
Urothelial papilloma
Papillary urothelial neoplasm of low malignant potential (PUNLMP)
Low-grade papillary urothelial carcinoma
High-grade papillary urothelial carcinoma



2.3. Risk prediction models for bladder cancer

While the tumor grade alone has prognostic value, so do many additional clinical variables. The EORTC and CUETO are two well studied risk assessment models which are used for prognostication of NMI bladder cancer. Both incorporate the WHO 1973 tumor grade along with multiple clinical variables in order to predict the recurrence and progression risk of a patient. The EORTC risk table was developed by the European Organization for Research and Treatment of Cancer based on a meta-analysis of a study population of 2 596 patients with Ta or T1 bladder cancer, for which the tumor stage, grade, size, concomitant CIS, recurrence history, and multifocality were shown to be independently associated with recurrence and progression [29]. The variables were given weights based on their predictive value, and by summing the score the patients are categorized into one of four risk groups with different probabilities of recurrence and progression. The Club Urologico Español de Tratamiento Oncologico (CUETO) collaborative research group published a meta-analysis of 1 062 patients to evaluate recurrence and progression risk assessment in a cohort where more patients were given installations of Bacillus Calmette-Guerin (BCG) rather than chemotherapy (See

section 2.6) compared to the EORTC study population [28]. The CUETO risk model uses different weights for the variables and also includes the variables age and gender. Based on the risk category, the EORTC and CUETO risk tables predict 5 year recurrence probabilities ranging from 31-78% (EORTC) and 21-67% (CUETO), and progression probabilities ranging from 1-45% (EORTC) and 4-34% (CUETO). The lower recurrence and progression risk seen in the CUETO risk table reflects the effectiveness of BCG treatment. The clinical application of either system requires knowledge of the underlying patient population due to the impact of treatment usage. There is also regional variations in the guidelines for NMI risk stratification, where the European Association of Urology (EAU, Europe), the National Institute for Health and Care Excellence (NICE, United Kingdom), the Canadian Urological Association (CUA, Canada), and the American Urological Association (AUA, United States) recommending the use of the risk stratification tables or variations thereof, while the National Comprehensive Cancer Network (NCCN, United States) recommends a stratification method based on the combination of stage and WHO 2004/2016 grade. A potential drawback of the established stratification methods is that many possibly prognostic variables are not yet incorporated due to a paucity of evidence. This includes clinical variables such as tumor stage at second look resection, histological variants, lymphovascular invasion, invasion depth in T1 [35], and a range of molecular markers including mutations, copy number alterations or gene expression signatures [64-67].

2.4. Histology

In addition to the pathological report of invasion depth and grade of the tumor, urinary bladder cancer also has a high propensity for divergent differentiation and shows a diversity of morphological features, as recognized in the WHO 2016 classification of bladder cancer [68]. High grade urothelial carcinoma can exhibit a number of variant histologies, including micropapillary, sarcomatoid, plasmacytoid, or nested variants and several others [69-72]. Several patterns are so distinctive that they are categorized as non-urothelial histological variants distinct from urothelial carcinoma. The majority is made up of squamous cell carcinomas (SCC), while other variants e.g. adenocarcinomas and neuroendocrine/small cell carcinomas are very rare. Between 10%-25% of bladder cancers are non-urothelial variant of bladder cancer, but the frequency varies strongly between geographic regions. In the western world more than 90% of bladder cancers are classified as urothelial carcinoma while ~3-5% are categorized as SCC, however in regions with a high prevalence of Schistosoma haematobium infections, such as East Africa and the Middle East, up to 75% of bladder cancers are squamous cell

carcinoma. Schistosoma haematobium is a flatworm whose larvae burrow into the skin and travel to the liver where they mature into adult flukes and then migrate to the bladder veins where they sexually reproduce and lay eggs in the bladder wall. These eggs cause chronic inflammation of the bladder wall, leading to squamous cell carcinomas. Chronic inflammation caused by urinary tract infections is also associated with an increased risk of squamous cell carcinoma. Primary adenocarcinoma is a rare histological variant, accounting for only 0.5-2% of bladder cancers, and stands out from other urothelial malignancies by its mucoid/glandular appearance. It can originate both from the urothelium as well as the urachus, the remnant of the channel between the bladder and the umbilicus, however most are secondary adenocarcinomas involving the bladder by direct extension or metastasis from other sites [73,74]. While rare, primary non-epithelial bladder malignancies does exist (e.g. sarcomas and lymphomas) with tissue origins such as the stromal, fat, or muscle layer of the bladder [75]. In most cases, however, non-epithelial tumors found in the bladder are secondary tumors originating from colon, prostate or female genital organs [73].

2.5. Multifocal bladder cancer

A prominent characteristic of urothelial carcinoma is the high frequency of both multifocal synchronous and metachronous tumors. Synchronous tumors are present at the same time (or arise within a few months) but in different locations in the bladder (i.e. not a direct recurrence), while sequential new tumors that arise later are described as metachronous. These types of tumors are thought to originate from a bladder field disease, wherein transformed cells spread throughout the bladder. It has been demonstrated that fields of tumor-adjacent morphologically normal urothelium, as well as premalignant lesions, hyperplasia, and dysplasia, show similar genetic alterations as the overt tumor to various degrees [76-83]. While metachronous tumors are clonally related, they often show patterns of genetic alterations that do not strictly coincide with the chronology of tumor appearance. An earlier occurring tumor can show more genomic changes than a later tumor, or the later tumor can harbor alterations that are incompatible with a direct clonal relationship [84,85]. Evidence suggests that mildly transformed premalignant cells gradually expand throughout the epithelium and from which overt tumors eventually develops [76,77]. Exactly how this expansion of premalignant urothelium occurs still remains unclear. Similar to intra-tumor heterogeneity, additional acquired alterations give rise to new expanding subclonal fields within the bladder. Synchronous and metachronous tumors will therefore often share early trunk mutations and genomic aberrations, while also containing multiple private alterations [86-88].

2.6. Treatment of bladder cancer

Because of the unpredictable disease course, high recurrence rate, and risk of progression, patients with bladder cancer require continuous, costly, follow-up monitoring which poses a burden both to the patient and the healthcare system [89,90]. This makes bladder cancer one of the most expensive malignancies per patient [91,92]. The tumor stage is closely related to patient outcome and is the crucial factor for treatment selection. For patients with stage Ta, Tis, and T1 tumors the preferred treatment choice is local resection of the tumor, paired most commonly with intravesical immunotherapy in the form of Bacillus Calmette-Guerin (BCG) instillations or intravesical cytostatic chemotherapy using mitomycin C, a DNA crosslinking drug, or DNA intercalating agents such as epirubicin or pirarubicin. Intavesical therapy is particularly well suited for NMI bladder cancers because of their superficial confinement as well as the anatomical properties of the bladder, and aims to reduce the risk of recurrence and progression. Chemotherapy has been shown to significantly reduce the rate of recurrences, but shows little effect on the rate of progression [93]. BCG was developed as a live attenuated vaccine against tuberculosis in the beginning of the 20th century. In the 1930s it was noted that tuberculosis patients had a lower cancer incidence [94], and BCG was proposed as a potential cancer therapy. The first use of BCG for the treatment of bladder cancer was reported in 1976 [95]. Intravesical chemotherapy and BCG both significantly reduces the rate of tumor recurrences, but the reduction is greater with the use of BCG. BCG also has the advantage of reducing the rate of disease progression, and is considered the superior treatment of choice both for high and intermediate risk NMI bladder cancer [96-104]. The use of BCG is primarily limited by the eligibility of the patients due to toxicity, underutilization by clinicians, as well as a recent supply shortage associated with its production [105]. The mode of action of BCG immunotherapy is complex and not fully understood, depending on molecular interactions between the patient, the immune system, and the tumor [106-109]. While BCG is effective, the treatment fails in up to 30-40% of patients [110,111]. It has been reported that the survival rate for patients progressing from NMI to muscle invasive disease may be worse than for patients with MIBC without a history of NMIBC [112]. The most appropriate treatment method for high risk T1 and operable primary or progressed muscle invasive tumors (stage ≥T2) is radical cystectomy (RC). This is a surgery with curative intent involving removal of the urinary bladder and pelvic lymphadenectomy, and is always accompanied by prostatectomy in males or usually hysterectomy in females. If the patient is eligible, the cystectomy may be paired by systemic neoadjuvant chemotherapy (NAC) to target potential micrometastatic disease prior to surgery, which has been shown to provide a 5-8% increase in overall survival (OS) and a 9% increase in cancer specific survival (CSS) [113-118].

The chemotherapy mainly consists of cisplatin-based combination e.g. gemcitabine and cisplatin (GC), or methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) [119]. These regimes are sometimes also used in the adjuvant setting.

2.7. Immune checkpoint inhibitors

By far the most impactful recent advancement in the treatment of bladder cancer and cancer in general has been immune checkpoint inhibitor (ICI) therapy [120-123]. In order to survive and proliferate, tumors often adopt immune escape strategies that protect them from being targeted by the immune system, which could otherwise react against the tumor cells. The increasing knowledge of the interaction between tumor and immune system has led to the development of this new class of drugs that disrupts this immune avoidance. In the human body approximately 150 billion cells naturally die daily, which are cleared out primarily by macrophages and dendritic cells of the immune system through phagocytosis. These are also referred to as professional antigen presenting cells (APCs), and form a link between the innate and the adaptive immune system by internalizing self- and non-self-antigens and processing these into peptides which are then displayed by the major histocompatibility complexes (MHC). Dendritic cells then mature and migrate to regional lymph nodes through the lymph vessel system. If the antigens that the dendritic cell is presenting are immunogenic they can interact with and prime T cells by inducing expansion and maturation into effector T cells. While tumors originate from host cells, they can be immunogenic due to mutated or incorrectly processed proteins [124,125]. However, like normal cells they have a range of surface membrane receptors that interact with surface membrane receptors on cells of the immune system which provides both stimulatory and inhibitory signals. The signals from these interactions function as balancing immune checkpoints, crucial for the maintenance of self-tolerance i.e. the avoidance of autoimmunity. While the above description is highly simplified, current immune checkpoint inhibitor therapy is largely based on targeting the receptors which provide the inhibitory signals stopping T cells from targeting the cancer cells, thereby inducing or restoring an anti-tumor immune response. Current checkpoint inhibitors mainly target the interaction between PD-1 and PD-L1, which are members of the Ig superfamily. PD-L1 is expressed on both hematopoietic and non-hematopoietic cells, while PD-1 is expressed on the surface of T cells. The PD-L1/PD-1 interaction leads to “T cell exhaustion” that impairs cytotoxic activity and decreases the effector cytokine production resulting in immune-response inhibition [126-128]. Five separate human/humanized IgG monoclonal antibodies which target the interaction between PD-1 and PD-L1 have

been approved for use in advanced bladder cancer by the US FDA between 2016 and 2017; Pembrolizumab (Keytruda, Merck and Co. Inc.) [129-131] and Nivolumab (Opdivo, Bristol-Myers Squibb Co.) [132,133] which targets PD1, and Atezolizumab (Tecentriq, Genentech Inc.) [134-137], Avelumab (Bavencio, EMD Serono Inc) [138], and Durvalumab (Imfinzi, AstraZeneca UK Limited) [139] which target PD-L1. The approvals span use in second-line or first-line for patients who have either failed conventional therapy or are ineligible for standard treatment. The clinical trials to date have showed response rates ranging between approximately 15-30%, with several patients achieving complete response [121-123]. The promising results seen in bladder cancer and many other malignancies have spurred an unprecedented research effort into checkpoint inhibitor therapy and immune modulating strategies, however a drawback of the current immunotherapies is that they are effective in only a minority of patients. The underlying tumor and host properties that govern response is a key field of study, as successful anti-tumor T cell activation is influenced both by a range of genomic properties of the tumor, such as mutation load [140], as well as the complex interactions within the tumor microenvironment composed of a variety of immune and stromal cells and secreted factors in addition to tumor cells. The terms “hot” and “cold” is often used to describe tumors with an immunogenic or non-immunogenic microenvironment [141,142]. Biomarkers that reliably identify highly immunogenic tumors or predict response are actively searched for, as are ways to increase the immunogenicity of a “cold” tumor which could extend the benefits of checkpoint inhibition therapy to more patients. Several large phase III trials are ongoing in bladder cancer (e.g. IMvigor130 (NCT02807636), DANUBE (NCT02516241), KEYNOTE-361 (NCT02853305), CheckMate-901 (NCT03036098), and JAVELIN Bladder 100 (NCT02603432)) with enrollment goals of around 1000 patients each. Multiple studies are investigating PD-1 or PD-L1 inhibitors in combination with other immune checkpoint inhibitor (e.g. Tremelimumab or Ipilimumab targeting CTLA-4), other forms of immunomodulatory agents, or together with targeted therapies [122,143-145]. Checkpoint inhibitors are also being evaluated for their safety, efficacy, and interaction with BCG in patients with high grade NMIBC (e.g. NCT02324582, NCT02808143, NCT02792192, NCT02625961, and NCT02844816). Because ICI therapy is so strongly dependent on the immune system, it is likely that research results obtained from the wealth of ongoing ICI and immuno-oncological studies across many different cancer types [146-150] will have pan-cancer relevance for predicting response, increasing T cell activity, promoting an immunogenic microenvironment, overcoming resistance, managing side effects, prolonging responses, and ultimately improving the survival rate of patients.

2.8. Challenges in bladder cancer pathology

While pathology plays a central role in the clinical management of bladder cancer there are a number of challenges [151], as well as unmet needs particularly in regards to predicting therapeutic response. A long-standing concern is the variability in bladder tumor diagnosis among pathologists. Correct tumor grading is of importance for the treatment selection and quality of care for bladder cancer patients, but despite efforts towards standardization there remains high inter-observer variability between pathologists [46]. The quality of the TURBT procedure and preparation of the tissue sample can also influence the pathological interpretation. The TURBT specimen should include the underlying bladder wall with the detrusor muscle in order to adequately determine the depth of invasion. If the specimen does not contain muscle, which reportedly can occur in up to 50% of TURBT specimen, then the pathologist cannot determine whether the tumor is muscle invasive [152]. For this reason it is recommended that a "second-look" TURBT is performed some weeks after the initial procedure [153,154]. The importance of second-look resection has been demonstrated by the finding that upstaging of T1 tumors to MIBC, with resulting change of treatment, is common [155,156], and that recurrence- and progression rates of NMI tumors is lower if second-look resection was performed [154,157]. Many of the more subjective aspects of bladder cancer prognostication could be improved through better diagnostic tools. Molecular diagnostics are a routine part of the clinical management of many cancers and are important both for prognostication and treatment selection. Molecular analyses have been largely absent from the management of bladder cancer, with current risk stratification and treatment selection relying on clinical and pathological parameters, which are inadequate for predicting response to BCG, NAC, and immune checkpoint inhibitor therapy [121,158,159]. The established pathology will need to be complemented by refined molecular diagnostics and suitable biomarkers in order to better predict, explain, and expand therapeutic response. There is also a need for objective quantitative molecular markers for risk stratification. Robust urinary biomarkers could potentially improve the clinical management of bladder cancer by reducing the reliance on cystoscopy, while liquid biopsies such as circulating cell-free DNA analysis could be used as a non-invasive method of disease course monitoring [160-163]. In a clinical trial setting, the evaluation of emerging therapeutic agents can benefit from utilizing a molecular approach [164]. Bladder cancer research has historically lagged behind that of many other forms of cancer partly due to a lack of research funding, disproportionate to its high mortality rate and cost of care, as well as the low general awareness of the disease [165-167]. Cost-efficient new methodologies and the advent of novel immunotherapy agents has led to rapid changes in the field, providing both a greater insight into the molecular biology of bladder cancer and opening up new options for treatment.

3. Molecular characterization of bladder cancer

Advancements in molecular characterization technologies such as PCR, microarrays, and DNA and RNA sequencing have enabled in-depth characterization of the molecular changes that are involved in the initiation and progression of bladder cancer, but have also highlighted vast complexity, heterogeneity, and adaptability. Molecular characterization has resulted in an expansion of the available armamentarium for many forms of cancer. The success of targeted treatment in chronic myeloid leukemia with BCR-ABL gene fusion using Imatinib showed that genetic alterations could be potent therapeutic targets. In solid tumors, *ERBB2* amplified breast cancer respond well to anti-ERBB2 antibody therapy using Trastuzumab, while small molecule receptor tyrosine kinase (RTK) inhibitors such as Erlotinib and Gefitinib are effective in the treatment of *EGFR* mutated lung cancer. In contrast, despite extensive molecular characterization of bladder cancer, no effective targeted therapy has yet made it into clinical routine.

3.1. Mutations

Bladder cancer displays a wide range of frequently occurring somatic DNA alterations, including both mutations and copy number alterations. Bladder cancer is among the most highly mutated cancers, with an average overall mutation burden of 7.7 mutations per megabase and 302 exonic mutations per tumor, with only lung cancer and melanoma showing higher mutation rates [168-171]. Early studies identified that non muscle invasive tumors have a very high rate of activating mutations in fibroblast growth factor receptor 3 (FGFR3) [172-176] and in PI3-kinase catalytic subunit (PIK3CA) [177,178], seen in between 50-70%, and 15-25% of tumors, respectively. Frequent inactivating mutations were found in tumor suppressor genes such as *TP53, RB1,* and *TSC1*. Recent studies of large cohorts examined using genome or exome wide sequencing techniques have greatly expanded the known repertoire of activating and inactivating mutations [170,171,179-182]. The majority of recurring somatic mutations occur in genes involved in

mitogenic signaling, cell cycle regulatory pathways, or chromatin modification (Table 2). The main mitogenic pathways affected include the PI3K/AKT/mTOR pathway with activating mutations in *PIK3CA*, *PIK3R1*, *PIK3R2*, *MTOR* and *AKT1*, and inactivating mutations in pathway regulators such as *NF1*, *PTEN*, *TSC1* and *TSC2* [183]. Among the cell surface receptor tyrosine kinases (RTKs) that activate these pathways, mutations in *FGFR3* are by far the most frequent, but mutations are also seen in members of the epidermal growth factor receptor family including *EGFR*, *ERBB2*, and *ERBB3*. Activating mutations within the MAPK/ERK pathway are less common [182] but can occur in various members of the mitogen-activated protein (MAP) kinase family, as well as in *RAF1*, *BRAF*, *HRAS*, *KRAS*, and *NRAS*. Growth factor-mediated signaling or mutational activation of these interconnected pathways can influence a broad range of cellular processes, including cell growth, cell cycle progression and proliferation, protein translation and synthesis, and regulation of apoptosis [184-187]. In addition to mutations in the mitogenic pathways, several cell cycle regulatory checkpoint genes are also affected by mutations or copy number alterations, the most common being inactivating *RB1* mutations, which disrupt the regulation of members of the E2F transcription factor family [188], and *CDKN2A* genomic deletions. Inactivating mutations also occur in the *FBXW7* gene, which encodes a protein involved in ubiquitin mediated degradation of cyclin E [189]. *TP53* is the most frequently mutated gene involved in cell cycle arrest and DNA damage response, and is the overall most frequent mutation in muscle invasive bladder cancers [190-192], where roughly 50% of tumors carry inactivating mutations [170]. Mutations are also found in DNA damage detection genes, such as inactivating mutations of *ATM* which interferes with P53 degradation and DNA damage induced activation, and in DNA damage repair genes such as the excision repair cross-complementation group 2 (*ERCC2*).

The overall most common mutation target is the promoter region of the Telomerase Reverse Transcriptase (*TERT*) gene, seen in up to 70% of bladder cancers [193-196]. The mutations lead to increased TERT expression which results in avoidance of cell division induced senescence (Hayflick limit) by maintaining telomere ends. Mutations are also highly frequent in the Stromal Antigen 2 (STAG2) gene located on the X chromosome. STAG2 is a member of the cohesin complex and approximately 10-30% of NMIBC and 10% of MIBC harbor inactivating mutations [170,180,182,197-199]. *STAG2* mutations have been linked with aneuploidy in vitro [200], but no clear association between *STAG2* mutations and genomic instability was seen in bladder cancer [199], and the significance of *STAG2* mutations remains controversial [201]. The high mutation frequency in genes like *TERT*, *PIK3CA*, and *FGFR3* in NMIBC make them attractive biomarkers for bladder cancer detection [202,203].

A recent discovery is the high frequency of mutations in a set of genes involved in chromatin remodeling, including the histone methyltransferases *KMT2D* (MLL2), *KMT2C* (MLL3), *KMT2A* (MLL1), the histone demethylase *KDM6A* (UTX), the histone acetyltransferases *EP300* and *CREBBP*, as well as *ARID1A* which is a part of the ATP-dependent chromatin remodeling SWI/SNF complex (SWItch/Sucrose NonFermentable, a nucleosome-remodeling complex) [170,171,179-182]. Chromatin remodeler mutations were shown to be frequent both in NMI and MI bladder cancer. Mutations of chromatin remodeling genes are found in multiple types of cancer; however their exact effect is not yet clearly understood. As epigenetic chromatin modifications play a central role in both transcriptional regulation and maintenance of genomic stability, dysregulation of these histone modifying enzymes likely promotes malignant transformation by broadly altering chromatin accessibility which could cause aberrant gene expression or defective differentiation.

Several developmental pathways show mutations, including genes in the sonic hedgehog (SHH) pathway (*PTCH1*), the WNT pathway (*APC* and *CTNNB1*), as well as the PPARG/RXR signaling pathway (*RXRA*), and other transcription factors involved in bladder development (*ELF3* and *KLF5*). Many of these mutations are still poorly characterized and their exact role in bladder cancer remains under investigation [204-213].

Table 3. Approximate mutation frequencies in NMI and MI bladder cancer. Assembled from references; [170,179-182,193,194,197-199,214-220]

		Approximate Mutation Freq.	
Pathway/Process	**Mutation type**	**NMI**	**MI**
RTK			
FGFR3	Activating mutation	50-70%	10-20%
EGFR	Activating mutation	<5%	<5%
ERBB2	Activating mutation	8%	5-10%
ERBB3	Activating mutation	10	5-10%
PIK3/AKT/mTOR			
PIK3CA	Activating mutation	20%	20%
AKT1/2	Activating mutation	1-5%	1-5%
NF1	Inactivating mutation	20%	8%
TSC1	Inactivating mutation	10-15%	8%
TSC2	Inactivating mutation	5%	3%
PTEN	Inactivating mutation	3%	3%
PIK3R1	Inactivating mutation	1-9%	1%
MAPK/ERK			
RAF1	Activating mutation	1-2%	1-2%
BRAF	Activating mutation	1-2%	1-2%
HRAS	Activating mutation	3-5%	5%
KRAS	Activating mutation	3-5%	5%
NRAS	Activating mutation	1-2%	1-2%
Cell Cycle Progression			
RB1	Inactivating mutation	7%	15%
FBXW7	Inactivating mutation	NA	8%
TP53/DNA damage			
TP53	Inactivating mutation	8%	50%
ATM	Inactivating mutation	15%	15%
ERCC2	Inactivating mutation	NA	10%
Telomere maintenance			
TERT	Promoter Mutation	60-80%	60-80%
Histone Modification And Chromatin Remodeling			
KMT2A (MLL1)	Inactivating mutation	18%	11%
KMT2D (MLL2)	Inactivating mutation	12%	28%
KMT2C (MLL3)	Inactivating mutation	19%	18%
KDM6A (UTX)	Inactivating mutation	22%	26%
EP300	Inactivating mutation	15%	15%
CREBBP	Inactivating mutation	12%	12%
ARID1A	Inactivating mutation	13%	25%
Cohesin Complex			
STAG2	Inactivating mutation	18-36%	14%
Developmental Pathways			
RXRA		5%	5%
ELF3		8%	12%
CTNNB1	Inactivating mutation	1-2%	1-3%
APC	Inactivating mutation	2-4%	5-15%
PTCH1	Inactivating mutation	NA	5%
KLF5	Inactivating mutation	NA	6%

3.2. Mutational signatures

Somatic mutations often occur widely dispersed throughout the genome, but with patterns, or "signatures", associated with the underlying mutational mechanism. Through exome or whole-genome sequencing, the patterns of C>A, C>G, C>T, T>A, T>C, and T>G substitutions in 96 different trinucleotide context has been investigated in thousands of cancer genomes. A large number of mutation patterns have been described [168,221-224], and curated into 30 distinct mutational signatures [225] with distinct distribution and enrichment of mutations in specific trinucleotide sequence contexts. The aetiology of many signatures has been described, such as defects in DNA repair genes (e.g. *BRCA1/2* or *ERCC2* mutations), tobacco smoke exposure, or UV exposure. Tobacco smoking is the leading risk factor associated with bladder cancer due to accumulation of carcinogens (e.g. aromatic amine compounds and polycyclic aromatic hydrocarbons) in the urine. Both in tissues directly exposed to tobacco smoke (i.e. lung) and those with secondary exposure like the urothelium, these carcinogens can trigger carcinogenesis through the formation of DNA adducts and subsequent base substitutions. The Signature 4 mutation pattern is associated with tobacco smoke exposure and is characterized by C>A transversions, with a minor contribution from other base substitution classes. This signature is frequent in lung cancer and in other cancers arising from epithelium exposed to tobacco smoke. Despite the association between smoking and bladder cancer, this particular mutation pattern is not evident in bladder tumors [226]. The four most prominent mutational signatures in bladder cancer instead include Signatures 1, 5, 2, and 13 [170]. Signature 1 correlates with age of cancer diagnosis and is the result of endogenous mutations caused by spontaneous accumulation of 5-methyl-cytosine deamination events (C>T in CpG context). Signature 5 is characterized by a broad pattern of mutations across all trinucleotide variants, and also shows an association with age; however it has also recently been associated with mutations of the nucleotide excision repair gene *ERCC2* [170,227,228]. The strongest mutational signatures in bladder cancer are the two related Signatures 2 and 13, which arise from the deaminase activity of the endogenous apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) proteins [169,229]. Specifically, the APOBEC3A and APOBEC3B proteins bind single stranded DNA (ssDNA) and induce a mutation spectrum consisting of C>T and C>G mutations in a T**C**W (A/T) motif context [224]. They are normally involved in restriction of retroviruses and retrotransposons through C-to-U editing [230], but can target transiently exposed ssDNA under circumstances such as double-stranded breaks, transcription, DNA repair, and DNA replication [231]. APOBEC induced mutations are frequently seen at chromosomal aberration breakpoints where ssDNA may have been exposed by strand breaks, however there is also evidence that APOBEC3 deamination could have a causal role for DNA breaks and

genomic instability [232]. The expression level of APOBEC3A and APOBEC3B has been shown to correlate to the degree of APOBEC induced mutations [170,229]; however the mechanisms that lead to overexpression in tumors is still under investigation [233]. The degree of APOBEC mutational signature as well as overall mutation load and mutation process types has been linked with better survival outcome in the TCGA bladder cancer dataset [170,234]. Broad quantification of the mutation level of a tumor is a promising biomarker, as the overall mutation load is associated with response to immune checkpoint inhibitors, which is likely because of an increased number of neoantigens [164].

3.3. Copy number alterations

In addition to mutations, the major pathways are also frequently disrupted through genomic amplifications and deletions. The earliest investigations of the genomes of bladder cancer used cytogenetic karyotyping based on the chromosome-specific banding pattern made visible through the use of dyes during the mitotic cell division. Through visual inspection of chromosomes in tumor cells, the early cytogenetic karyotyping studies revealed non-random chromosomal alterations of chromosomes 1, 3p, 7, 9, 11p, and 13, as well as isochromosomes 5p and 8q [235-238]. The method is limited to very large genomic alterations, such as whole chromosome or arm level events, and most individual studies used cohorts of limited size. Still, the broad overall pattern of chromosomal alterations seen in bladder cancer was accurately captured in subsequent review articles of the field [239]. Despite the low resolution of cytogenetic karyotyping, it guided the early identification of some of the most frequently altered genes in UC, such as genomic loss of heterozygosity (LOH) of the cyclin dependent kinase inhibitor *CDKN2A* (9p) [240], *RB1* (13q) [241], and *TP53* (17p) [242]. Many subsequent discoveries of targets of genomic aberrations were made possible through the use of comparative genome hybridization (CGH), where DNA from the tumor is fluorescently labeled and hybridized with DNA from a normal tissue such as blood, labeled with a different fluorescent dye [243]. The ratio between the two dyes is then examined along the metaphase chromosomes to detect gains and losses. The increased resolution of this methodology allowed for detection at the cytoband level, including alterations at 6p22, 8q21, 13q21-q34, 1q31, 3q24-q26, and 1p22 [244]. The CGH methodology was refined through the use of DNA microarrays, using libraries of bacterial artificial chromosomes (BAC) containing human DNA fragments, with known genomic positions and covered genes, which were generated by the Human Genome Project. Early genome wide arrays contained roughly 1000-3000 genomic BAC clones giving a resolution of roughly 1-2 Mb which allowed detection of cytoband level alteration [245-247].

High resolution arrays with ~30 000 BAC clones were used to investigate large tumor cohorts of all stages, producing detailed whole genome maps of frequently occurring alterations. These studies could also confirm the large difference in the extent and pattern of genomic alterations that exists between G1, G2, and G3 tumors, and between Ta, T1, and ≥T2 tumors [245,248-250]. It also enabled very precise mapping of narrow alterations such as amplifications of the *E2F3*/*CDKAL1*/*SOX4* 6p22 locus by Heidenblad et al. [250], which is found in up to 20% of bladder cancers and is particularly common in aggressive tumors [251,252].

Whereas muscle invasive tumors often show extensive genomic instability, low-grade low-stage tumors have relatively few genomic alterations [248] (Figure 2). The most common alteration is deletion of chromosome 9, seen in >50% of both NMIBC and MIBC. Loss of chromosome 9 and *CDKN2A* stands out as an early event in the development of bladder cancer, as it is one of the few genomic events associated with low-grade low-stage tumors. The *CDKN2A* gene is one of the primary deletion target on the p-arm of chromosome 9, with hemizygous deletions in >50%, and homozygous deletion in 15-30% of tumors. Loss of the *CDKN2A* tumor suppressor gene impacts both cell cycle regulation and the *TP53* pathway, as it encodes both the P16 and the $P14^{ARF}$ protein depending on the reading frame [253]. The P16 protein binds and inhibits CDK4/6 from phosphorylating RB1, preventing G1/S transition, while the $P14^{ARF}$ protein functions as a P53 activator by sequestering MDM2, a primary P53 regulator. The *MDM2* gene is itself a frequent target of genomic alterations, with amplifications in ~5% of tumors. The recurring alterations broadly affect the same pathways as the gene mutations, such as cell cycle regulation (e.g. *CCND1* and *E2F3* amplifications and *RB1* deletions) and mitogenic signaling (e.g. *FGFR3*, *ERBB2*, *EGFR*, *RAF1* amplifications and *PTEN* deletions). From studies that utilize multi-level data (e.g. genomic copy number, mutation, methylation, and gene expression) in large bladder cancer cohorts we know that while the overall frequency of individual alterations can be low, nearly every single tumor show some form of alteration or dysregulation within the major pathways [170,182].

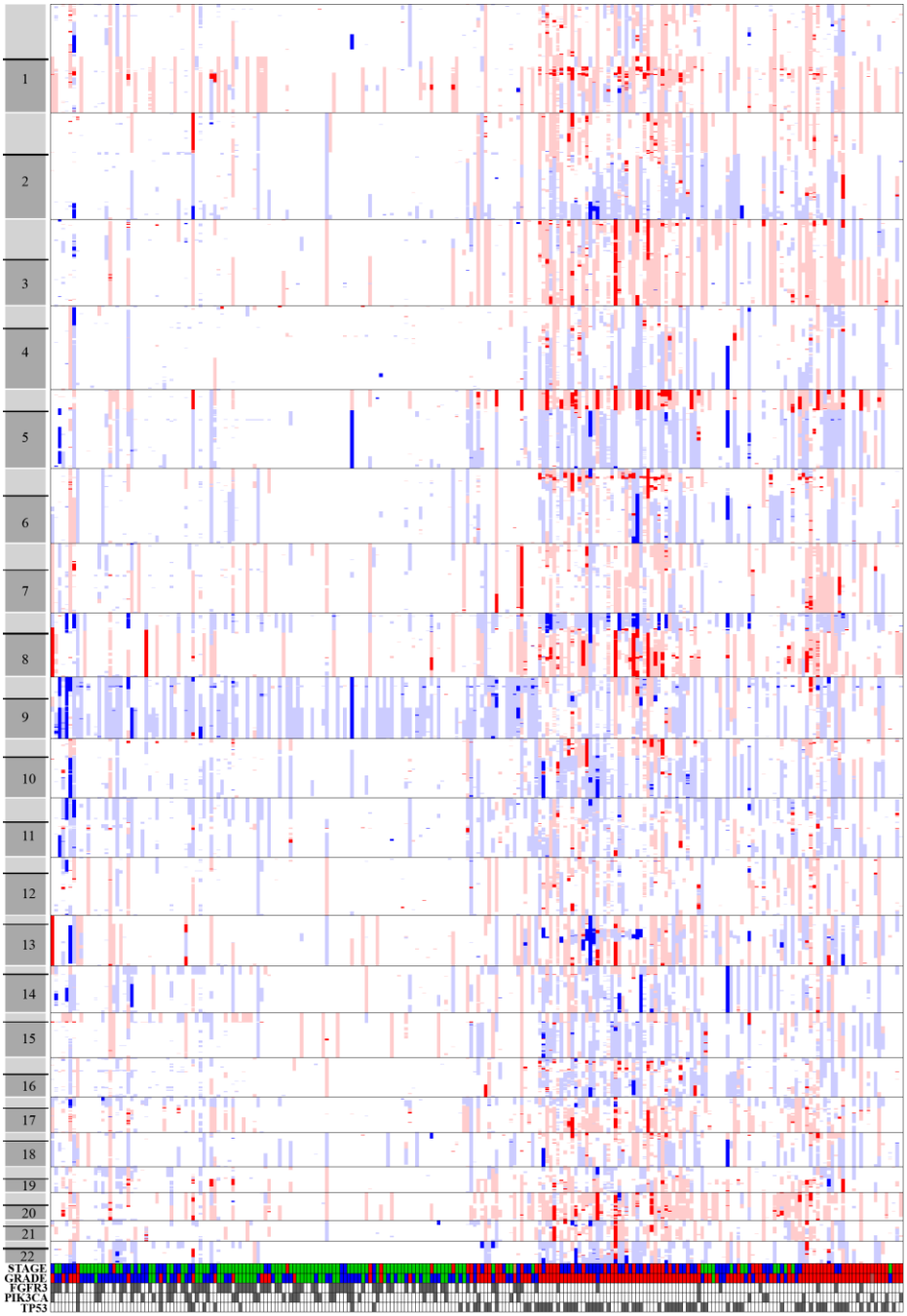


Figure 2. Illustration of the complexity and extent of bladder cancer genomic alterations. Lund data. Stage: (Ta, green), (T1, blue), (≥T2, red). WHO 1999 Grade: (G1, green), (G2, blue), (G3, red). Red, gain; blue, loss.

3.4. Models of UC development based on genomic alterations

Multiple models for the development of non-invasive and invasive bladder cancer have been proposed [254-256]. Based on the accumulated histological and molecular data, most models emphasize two distinct main pathways [174,175,255-260] wherein the normal urothelium becomes hyperplastic and through 9p/9q LOH and mitogenic mutations in *FGFR3*, *RAS*, or *PIK3CA* develops into papillary low-grade non-invasive tumors with a high recurrence rate (Figure 3). Invasive tumors are proposed to arise from carcinoma in situ or dysplastic urothelium though *RB1* and *TP53* alterations and genomic instability. Mutation of *TP53* and acquisition of additional genomic aberrations was proposed as events facilitating NMI to MI tumor progression. Studies have shown the large difference in the extent of genomic alterations between NMI and MI tumors [248] (Figure 2), which also corresponds to gene expression signatures of chromosomal instability [248,261]. While a two-pathway model corresponds well to the broad patterns of alterations seen in NMI and MI tumors, it is does not adequately describe the full complexity of how bladder cancer develops and progresses, or what gives rise to the multitude of histological variant and molecular subtypes that have been described.

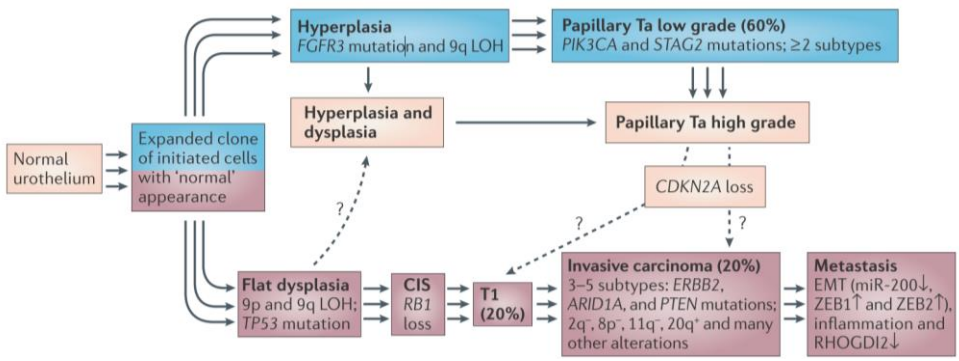


Figure 3. Illustration of an extended two pathway model of bladder cancer development. Reprinted from Knowles et al.(2015) [254] , with permission from Springer Nature.

3.5. DNA methylation

During morphogenesis and cellular differentiation cells adopt discrete mitotically stable phenotypes through regulation of gene expression levels. The study of epigenetics encompasses mechanisms which transduce the inheritance of gene expression patterns by adapting the chromatin without altering the DNA sequence. Epigenetic mechanisms function as dynamic heritable auxiliary regulatory components which act by controlling the expression of specific genes. The most prominent examples are DNA methylation and histone modifications. DNA methylation is a covalent addition of a methyl group to a cytosine nucleotide, which in differentiated human tissues occurs almost exclusively at 5'-3' CpG sites, a cytosine nucleotide followed by a guanine nucleotide in a 5'-3' direction, with non-CpG methylation primarily found in embryonic pluripotent cells [262]. DNA methylation is a dynamic modification and is introduced and maintained by methyltransferases (DNMT1, DNMT3A and DNMT3B) which uses S-adenosylmethionine (SAM) as a methyl group donor. Loss of DNA methylation can occur both passively through dilution over time if not maintained or through active methods such as oxidation and excision. DNA is wrapped around nucleosomes composed of eight histone proteins upon which a range of chemical modifications form a combinatorial "histone code" with transcription-regulatory functions [263]. The interplay of these epigenetic modifications is crucial for stimulating or repressing gene activity, and acts by regulating the functioning of the genome through changing the chromatin architecture [264]. During cell divisions DNA methylation is maintained through the genetic replication through the semiconservative propagation of DNA, while chromatin structure governed by histone modifications have been proposed to propagate through recycling of existing histones into the chromatin structure [265,266]. The bulk of epigenetic research in bladder cancer has focused on DNA methylation, whereas histone modifications have not been extensively investigated; however the frequent mutations seen in chromatin remodeling genes has emphasized the importance of this aspect of epigenetic regulation.

The human genome contains approximately 30 million CpG sites, of which up to 70-80% are methylated. The majority of methylated CpGs are located in repetitive elements such as SINE and LINE retrotransposons, LTR, and in pericentromeric satellite repeats. Unmethylated CpGs are mainly seen in CpG-islands, regions of densely clustered CpG-sites, around gene transcription start sites. Up to 75% of all gene promoters are located within a CpG-island region [267,268]. Early studies identified that methylation occurring in CpG-islands was linked with mitotically stable gene repression [269,270]. For this reason both research efforts and methodological development focused on promoter CpG-island methylation, and

the role of DNA methylation was for a long time mainly portrayed as a mechanism of gene suppression. Technological advancements such as whole-genome bisulfite sequencing have revealed a much more sophisticated and complex role for DNA methylation, in which gene silencing is only one facet [265]. The function of DNA methylation extends to gene activation [271,272], splicing regulation [273], nucleosomes positioning and chromatin structure [274,275], and recruitment of transcription factors [276]. Intergenic CpG-islands are actively investigated for their role in regulating enhancer activity and the expression of long non-coding RNA (lncRNA), micro RNAs (miRNAs) and other non-coding genes [277-279]. The major mechanism by which DNA methylation exerts these function is by modulating the chromatin access of transcription factors and the basal transcriptional machinery. Large-scale studies profiling the epigenome have shed greater light on the role of methylation and histone modifications in tissue development and differentiation, and uncovered tissue-specific differential methylation patterns [280-284].

Research into the fundamental mechanisms of epigenetic regulation is not specifically motivated by their role in disease and carcinogenesis, however the biological effect that epigenetic mechanisms exerts in morphogenesis and differentiation are directly relevant to cancer, which at its core is a disease resulting from the failure to adhere to normal cellular differentiation programs and activation of others [285]. Defects and perturbations in DNA methylation has been observed in nearly all forms of cancer, and can result in silencing of tumor suppressor genes and cause misregulation of multiple cell cycle, DNA repair, and chromosome stability genes, and hence contribute to genomic instability [286]. The research into epigenetic changes in bladder cancer has primarily focused on DNA methylation, and can broadly be divided into biomarker discovery for risk stratification and detection or genomic characterization of aberrant methylation patterns.

Early studies focused on the methylation status of smaller sets of candidate genes, such as tumor suppressors, but this expanded to an increasingly genome-wide scale with the development of methylation microarrays (e.g. Illumina 27k, Illumina 450k, and Illumina 850k EPIC bead arrays) and bisulfite sequencing. Studies identified significant differential methylation between normal and cancer tissue [287-289], as well as between different stages and grades of UC [290-293]. In line with the field disease effect, epigenetic changes reminiscent of those in the tumor have also been observed in tumor adjacent morphologically normal urothelium [292]. The stability of DNA methylation makes it a suitable candidate for use as a biomarker. It can readily be analyzed in fresh tissue samples, but also in formalin-fixed paraffin-embedded tissue, blood, and urine sediment cells.

Several studies have investigated the use of methylation changes as potential biomarkers for detection and prognostication [294]. The main approach is the use of methylation-specific PCR or bisulfite conversion based sequencing methods, targeting panels of genes that show differential methylation in tumors. Detection and classification of UC using urine samples from patients is one of the major clinical applications where methylation assays could be of importance. Much work in this field has been done by Zwarthoff and colleagues [295-298]. They recently reported a bladder cancer detection assay that utilized both methylation status of *TWIST1*, *ONECUT2*, and *OTX1*, and mutation status of *TERT*, *PIK3CA*, *FGFR3*, *HRAS*, *KRAS* and *NRAS*, as well as the clinical variable age. The assay had 93% sensitivity and 86% specificity, with a negative predictive value of 99% in patients with hematuria [202,203]. A similar approach also achieved a negative predictive value of up to 99% [299], which suggests that these type of urinary tests, if fully validated, could help identify many patients where cystoscopy examinations are not needed. Tumor stage and grade can influence the results of these tests, and sufficient DNA is not always obtained. In a cohort of 1239 NMIBC patients, van Kessel et al. also recently demonstrated that three progression risk categories could be defined based on *FGFR3* mutations and *GATA2* methylation [300]. Multiple urinary proteins have also been investigated as putative biomarkers for similar purposes, however a systematic review examining their specificity and sensitivity concluded that none have yet reached a level of accuracy warranting clinical implementation when compared to cystoscopy, and that protein based tests are likely to be superseded by DNA/RNA-based markers [301].

While utilizing methylation patterns at specific sites as a biomarker is relatively straight-forward, interpreting the function and biological effect of DNA methylation, and by extension also histone modifications and transcriptional regulation, on a genome-wide scale is immensely more complex. Lauss et al and Aine et al have showed the presence of distinct “epitypes” of bladder cancer, which differed in terms of stage, grade, and mutational status of *FGFR3* [290,291]. Three general DNA methylation patterns was reported which showed distinct associations with genomic features, CpG context, chromatin states, gene expression subtypes, and regulatory factor binding potential [290]. Notably, a pattern of differential anterior and posterior HOX gene methylation and gene expression was observed, which had associations with gene expression subtypes and survival. This pattern has since been identified in multiple independent cohorts [302,303].

3.6. Targeted therapy based on genomic alterations

Alterations of FGFR3, ERBB2, EGFR, and the mTOR pathway have long been recognized in subsets of bladder cancer. Trials investigating targeted therapy against these commonly altered pathways have to date not demonstrated a strong broad efficacy. One of the underlying causes for this may be the molecular heterogeneity that is observed in bladder cancer, or that tumors have a lower than expected dependence on specific oncogenic drivers. Several studies have demonstrated that therapeutic response of both targeted therapy, chemotherapy, and immune checkpoint therapy shows association with various molecular alterations or gene expression patterns. This suggests that molecular profiling will be of importance both for patient stratification and to enable post-hoc analysis of trials to explain response and non-response. Several trials using targeted therapy in combination with immune checkpoint inhibitors have been initiated, which may increase the efficacy over either treatment alone.

3.7. FGFR3 alterations

FGFR3 mutation or overexpression is seen in the vast majority of NMI bladder cancers, with a mutation frequency of around 10-20% in muscle invasive disease. The high frequency of *FGFR3* alterations makes it an attractive biomarker and potential drug target [304]. Knock-down of FGFR3 expression in *FGFR3* mutated cells, but not in normal cells, was shown to decrease proliferation and clonogenicity [305-307]. The FGFR1-3 kinase inhibitor BGJ398 was evaluated in a basket trial of solid tumors with FGFR alterations, which included 12 patients with bladder cancer who failed platinum-based chemotherapy. Among the 8 patients receiving ≥100 mg dosage 3 achieved a partial response and 3 achieved stable disease, warranting further investigation of BGJ398 in bladder cancer [308]. A number of FGFR directed therapies are currently under investigation in advanced urothelial carcinoma and other *FGFR* altered solid tumors; JNJ-42756493 (Erdafitinib, a pan-FGFR tyrosine kinase inhibitor) [309,310], LY3076226 (a FGFR3 directed antibody conjugated with DM4, a microtubule inhibitor), CH5183284 (Debio 1347, a FGFR1-3 tyrosine kinase inhibitor) [311,312], and BAY1163877 (Rogaratinib, a FGFR1-3 tyrosine kinase inhibitor) [313,314], as well as the FGFR3 directed monoclonal antibody B-701 [315] alone or in combination with immune checkpoint therapy (NCT03123055). Based on early results from the BLC2001 trial (NCT02365597), showing promising response rates in FGFR3-altered advanced bladder cancer [316], Erdafitinib was recently granted a “breakthrough treatment” designation by the FDA.

3.8. ERBB2, ERBB3, and EGFR alterations

Considerable research efforts have been devoted to exploring treatment options of bladder cancers with mutations, amplifications, or overexpression of members of the ERBB receptor family. Bladder cancer show genomic alterations and overexpression of ERBB2 (HER2) at a frequency comparable to that seen in breast cancer [317-319]. In breast cancer, the anti-ERBB2 monoclonal antibody Trastuzumab has proved a highly effective treatment against *ERBB2* amplified tumors (HER2-positive tumors) [320,321], as has other ERBB family directed antibodies (Pertuzumab) [322], antibody-drug conjugates (Ado-Emtansine Trastuzumab T-DM1) [323,324], and small molecule inhibitors such as Lapatinib [325]. Therapeutic targeting of ERBB2 in bladder cancer has shown mixed results. A phase II study with 44 patients with HER2-positive advanced UC, treated with Trastuzumab, gemcitabine, carboplatin, and paclitaxel, reported an overall response rate of 70% (5 complete and 25 partial), but with high toxicity [326]. A subsequent randomized study was limited by an unexpectedly low number of patients with HER2-positive tumors (61/563, 13%) and failed to show a significant improvement in in progression-free survival, response rate, or overall survival [327]. The low number of tumors categorized as HER2-positive may have resulted from the use of highly stringent genomic amplification criteria utilized in HER2 testing of breast cancer, while the limited response may be dependent on the type and context of the *ERBB2* alteration [317,318]. Wülfing et al reported modest but encouraging improvements in overall survival using Lapatinib (an EGRF and ERBB2 tyrosine kinase inhinitor) in a second-line setting for patients with ERBB2 and/or EGFR overexpressing UC [328], however a subsequent study of 232 patients with ERBB2 and/or EGFR overexpressing UC, randomly assigned to receive either placebo or lapatinib, failed to show a significant benefit in terms of PFS or OS [329]. Afatinib, an irreversible inhibitor of ERBB2 and EGFR, was shown to prolong the progression-free survival in a subset of patients with metastatic platinum-refractory UC [330]. Notably, mutation and copy number analysis revealed that all five responders had *ERBB2* or *ERBB3* mutations or amplifications, compared to only 1 out of 18 among the non-responders. Preclinical results have also indicated that the antibody-drug conjugate T-DM1 could be a more effective form of therapy against ERBB2 overexpressing bladder cancer [331]. ERBB2 remains a potential therapeutic target under active investigation, although primarily in tissue-agnostic trials of HER2-positive solid tumors. Results from ongoing clinical trials utilizing anti-ERBB2 therapy combined with immune checkpoint inhibitors in breast cancer will be of interest also for the field of bladder cancer.

3.9. mTOR pathway alterations

Multiple molecular alterations in the PI3K/AKT/mTOR signalling pathway have been described, with a high combined frequency in both NMI and MI bladder cancer [170,182]. When including overexpression or alterations of upstream RTKs and loss of expression of regulators such as PTEN, the frequency increases further. The signaling pathway is involved in regulation of cell growth, survival and metastasis, and contains multiple potential targets for therapeutic intervention. Clinical trials using small molecule inhibitors of mTORC1, such as the rapalogs everolimus and temsirolimus, in advancer UC have not met their endpoints, however a subset of patients have achieved partial response with one patient showing a sustained complete response [332-335]. A reanalysis of the Milowsky et al. [333] study used whole genome sequencing and identified that the tumor of the complete responder harbored *TSC1* and *NF2* mutations, while two out of three additional patients harboring nonsense *TSC1* mutations showed minor responses (17% and 24% tumor regression) [336]. A study examining the effectiveness of everolimus and pazopanib in solid tumors reported that one patient with mTOR mutated metastatic UC showed an exceptional response lasting 14 months [334]. Occasional exceptional responders have been reported in other cancers [337,338], however the utility of everolimus will ultimately depend on the ability to identify those patients likely to benefit from the treatment. A basket trial evaluating the use of everolimus in cancers with *TSC1*, *TSC2* or mTOR mutations is currently ongoing (NCT02201212), and may help determine the factors that govern therapeutic response. Other therapeutic interventions aimed at the PI3K/AKT/mTOR pathway are being explored (e.g. AKT inhibitors [339,340], dual target therapies [341], PI3K inhibitors [342,343], and dual PI3K/mTOR inhibitors [344,345]), but have not yet demonstrated clinical efficacy.

Large scale tissue-agnostic basket trials such as NCI-Match (NCT02465060), TAPUR (NCT02693535), and the Novartis Signature program which directs patients to treatment arms based on genetic alterations may help identify which patients benefit from various targeted treatments, and show whether matching drugs to molecular targets results in meaningful response rates and improved patient outcome.

3.10. Mutations in DNA damage response and repair genes

Neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy is an established standard of care treatment for muscle invasive bladder cancer [346]. Neoadjuvant chemotherapy improves patient outcome, with meta-analysis indicating a 5-8% improvement in overall survival at 5 years [117,118]. A large number of patients does not respond to NAC, and suffers adverse effects from overtreatment, which has spurred research efforts into finding biomarkers predictive of response and non-response to NAC. Van Allen et al. performed exome sequencing on a cohort of 50 pretreatment TURBT specimens of 25 extreme responders and 25 non-responders and identified an enrichment of nonsynonymous mutations of *ERCC2* (9/25) exclusively in the responder group [347]. Similarly, it was found that mutations in one or more of the DNA repair genes *RB1*, *ATM*, or *FANCC* was associated with response to cisplatin-based chemotherapy [348]. Reanalysis of this cohort revealed an enrichment of *ERCC2* mutations in the responder group (8/20, 40%) compared to the non-responder group (2/28, 7%) [349]. In both cohorts *ERCC2* mutation was significantly associated with better overall survival [347,349]. It was also reported that *ERCC2* mutations conferred sensitivity to cisplatin and radiation in bladder cancer cell lines [350]. In a cohort of 100 patients (treated with platinum-based chemotherapy) dichotomized based on the presence/absence of mutations of 34 DNA damage response and repair (DDR) genes, the 47 patients harboring mutations in one or more of the DDR genes showed significantly improved progression-free survival and overall survival [351]. The patients with DDR mutations also showed a higher total number of mutations and more copy number alterations. Alterations in DNA damage response and repair genes may also be potential predictive indicators for checkpoint inhibitor response. In a mutation screened cohort of 60 patients with metastatic UC treated with atezolizumab or nivolumab there was a significant difference in response rate between patients with DDR wild-type tumors (19%) compared to patients whose tumors harbored known, or likely, deleterious DDR mutations (80%) or with DDR alterations of unknown significance (54%) [352]. The association between DNA repair deficiency and chemotherapy response has been observed in other forms of cancer using formalized scores based on measurements of genomic instability, such as loss of heterozygosity [353], telomeric allelic imbalance [354], large scale transitions [355], or a combination of these scores into a measurement of homologous recombination deficiency (HRD) [356,357], which is under investigation for a potential utility in bladder cancer (e.g. Myriad Genetic's myChoice HRD™ panel).

4. Gene expression profiling of bladder cancer

A major challenge of molecular oncology is how to interpret the summed biological effect of the diverse genetic aberrations and dysregulated cellular mechanisms that is seen in any given tumor. As the intermediary between DNA and proteins, the RNA transcriptome is the link between the molecular underpinnings and the cellular phenotype, and as such, global gene expression profiling is one of the most powerful tools available for biological characterization. Early studies in bladder cancer showed that low grade NMIBC could be distinguished from MIBC based on gene expression patterns [249,358-368]. Many of these studies presented mRNA expression signatures that were reported to have clinical prognostic value, e.g. predicting overall survival, disease-free survival, or progression, however these types of signatures have been found to be difficult to validate in independent datasets, often performing no better than chance [369,370]. In the seminal work in breast cancer by Perou and Sørlie, hierarchical clustering of global gene expression was used to derive distinct molecular subgroups which exhibited distinct gene expression patterns relating to a variety of biological processes and pathways, showed association with pathological variables such as ERBB2 and ESR1 expression, and differed in survival [371,372]. These papers and subsequent profiling efforts have shown that although cancer is the consequence of a diverse set of somatic mutations and epigenetic alterations leading to altered protein function and aberrant transcriptional patterns, tumor cells tend to adopt prominent expression profiles and converge into molecular subtypes, commonly with a strong relationship to their tissue of origin [373].

4.1. First generation of molecular classification of bladder cancer

4.2. The Lund University classification

Several groups have conducted molecular profiling in order to characterize the underlying biology of bladder cancer, which could help provide an explanation to the notable heterogeneity of both biological and clinical behavior. The first steps towards a molecular subtype stratification was taken by Lindgren et al. who analyzed the genome wide expression of 144 urothelial carcinomas of all stages [248,249]. The tumors divided into two molecular subtypes (MS1 and MS2) wherein most Ta tumors were of the MS1 type, muscle invasive tumors were mainly of MS2 type, and T1 tumors were of both MS1 and MS2 types. *FGFR3* mutations and overexpression was frequent in the MS1 group, while genomic alterations of *TP53* and *RB1* were associated with the MS2 group. The MS2 group showed significantly more copy number alterations, and that there was a distinction between WHO 1999 tumor grades 1 and 2, and between grades 2 and 3 [61,248,249]. This work was greatly expanded upon by Sjödahl et al. who analyzed gene expression profiles, protein expression, and mutations of an extended mixed stage cohort (n=308) with an approximately equal proportion Ta, T1, and ≥T2 tumors [374]. Through sequential splitting of the gene expression dataset seven subclusters were obtained, representing five tumor phenotype groups that were not strictly associated with pathological stage and grade. The subtypes were named Urobasal A (UroA), Urobasal B (UroB), Genomically Unstable (GU), and Squamous Cell Carcinoma-like (SCC-like). The fifth group was termed "Infiltrated" as the tumors in this group showed extensive immune and stromal infiltration which dominated their gene expression profiles. Tumors of this cohort (n=237/308) were further analyzed through extensive immunohistochemistry (IHC) staining [375]. UroA tumors were characterized by mutations and overexpression of *FGFR3*, *PIK3CA* mutations, and expression of genes associated with FGFR3 signaling, urothelial differentiation, and the early cell cycle. The UroA subtype was enriched for low stage and grade tumors, commonly with papillary growth patterns. As expected, LOH of chromosome 9 was the most common genomic alterations in these tumors, while *TP53* mutations were rare. They showed histological features similar to normal urothelium and retained a degree of normal-like urothelial stratification with a basal cell layer expressing basal markers such as cytokeratin 5 (KRT5) and cytokeratin 14 (KRT14), EGFR, and P-cadherin (CDH3), several layers of undifferentiated intermediate cells, and occasionally a differentiated luminal layer with umbrella-like morphology. The overall proliferation was low in these tumors,

and primarily occurred in the basal and supra-basal layer. The name "Urobasal" was later updated to "Urothelial-like" to better convey these features, while retaining the abbreviation "Uro" [376]. The UroB subtype also displayed a stratified morphology, similar to the UroA group, but were commonly muscle invasive, showed higher proliferation, and frequently showed homozygous loss of *CDKN2A* on chromosome 9. The expression of basal markers was markedly extended in these tumors. Despite the resemblance to UroA, the UroB subtype appears to be more aggressive and associated with worse patient outcome [303,374]. The tumors of the Genomically Unstable subtype were mainly high grade T1 or muscle invasive, and showed extensive genomic alterations including frequent focal amplifications (e.g. 1q21-24 and 6p22). These tumors did not express FGFR3 or KRT5, but commonly overexpressed ERBB2. Unlike the Urothelial-like subtypes, cell divisions were not constrained to the basal cell layer, occurring throughout the tumor without clear directionality. In line with these observations, they showed frequent alterations in genomic maintenance and cell cycle regulation, including *TP53* mutations, loss of RB1, and overexpression of *CDKN2A* (P16) and late cell cycle genes (e.g. *E2F3* and *FOXM1*). The SCC-like subtype also displayed markedly increased proliferation and frequent *TP53* mutations but is highly distinct, with frequent signs of squamous differentiation, no urothelial stratification, and high expression of basal keratins KRT5 and KRT14 throughout the tumor parenchyma. These tumors rarely express FGFR3 or ERBB2, but almost invariably express EGFR. They also express a broad signature of keratinization, and lose expression of genes involved in urothelial differentiation including *FOXA1* and *GATA3* as well as markers of differentiation such as uroplakins (UPKs) and KRT20, suggesting that this subtype may arise from grossly aberrant differentiation. It should be noted that while the Uro and GU tumors show mRNA expression of markers of differentiation, their immunostaining pattern in high stage tumors is largely incompatible with normal urothelial differentiation and stratification, indicating that only a form of "pseudo-differentiation" is occurring in these tumors. The SCC-like subtype has readily been identified in other subsequent molecular profiling studies of bladder cancer, but has commonly been referred to as a "Basal" subtype, largely due to the similarities with the breast "Basal-like subtype" and the expression of markers of the basal urothelial cell layer. However, as *de facto* basal cells express key genes involved in urothelial differentiation which are lost in these tumors, it was later agreed that this subtype was more appropriately described using the nomenclature "Basal-Squamous-like" (BaSq-like), and defined by *KRT5* and *KRT14* overexpression and loss of *FOXA1* and *GATA3* expression [376]. Notably, women with bladder cancer appear to have a higher prevalence of BaSq-like tumors.

4.3. University of North Carolina (UNC), MD Anderson Cancer Center (MDA), and The Cancer Genome Atlas (TCGA) classification

Following the Lund publications, three classification studies on mainly MIBC tumors were published in 2014 by groups affiliated with University of North Carolina (UNC) (meta-dataset of 262 tumors from four cohorts) [377], MD Anderson Cancer Center (MDA) (cohort of 73 tumors) [378], and the TCGA consortium (cohort of 131 tumors) [171], which emphasized the major division seen between "Luminal-like" and "Basal-like" tumors. The UNC group classified tumors into two categories, Luminal and Basal, whereas the MDA group obtained a 3-group separation of Luminal and Basal tumors, and a third group that was named "p53-like". The TCGA study generated extensive multilevel data and found four gene expression clusters (Cluster I-IV). The MDA & UNC Luminal groups and the TCGA Clusters I and II all showed expression of urothelial differentiation transcription factors (e.g. *FOXA1*, *GATA3*, and *PPARG*), markers of differentiation (e.g. UPKs and *KRT20*), as well as *FGFR3* and *ERBB2*. Tumors in the TCGA Cluster III and the Basal groups of MDA and UNC were primarily those expressing *KRT5* and *KRT14* and losing *FOXA1* and *GATA3* expression, i.e. the BaSq-like subtype. Similar to the Lund Infiltrated group, the dominating feature of TCGA Cluster IV and the MDA p53-like subtype was immune and stromal infiltration.

4.4. Relationship between the Lund, TCGA, MDA, and UNC classification

Despite using slightly different methodologies, tumor groupings, and nomenclatures there were broad similarities between the first generation of Lund, MDA, UNC, and TCGA classifications. Aine et al. provided an extensive clarification of the interrelationship between the different stratification systems by applying the different classification systems to an expanded set of TCGA tumors (n=234), which indicated that they in essence capture similar biological themes although at different levels (Figure 4) [379,380]. The primary separation occurs between tumors that show expression of urothelial markers mentioned above (e.g. UNC Luminal, MDA Luminal and a proportion of p53-like, TCGA Cluster I and a majority of Cluster II, and the Lund Uro and GU subtypes), and those which lose expression of these genes (most frequently BaSq-like tumors). Categorizing tumors only as Luminal or Basal is insufficient to describe the molecular heterogeneity. Aine et al. identified an infiltrated mesenchymal (Inf-Mes) group

that showed signs of epithelial-to-mesenchymal transition (EMT) rather than a BaSq-like divergent differentiation, and identified a subset of tumors that express markers characteristic of Small-cell/Neuroendocrine tumors [380]. Lund UroB classified tumors frequently group with BaSq-like tumors, despite retaining expression of FGFR3 and urothelial markers, due to their high expression of keratinization related genes. A group of tumors with urothelial-like expression patterns but with high expression of genes relating to non-tumor cell infiltration was also identified in the dataset. This group was termed "Infiltrated-Epithelial" and corresponded well with the Lund "Infiltrated" and MDA p53-like groups, and showed overlap with TCGA cluster II.

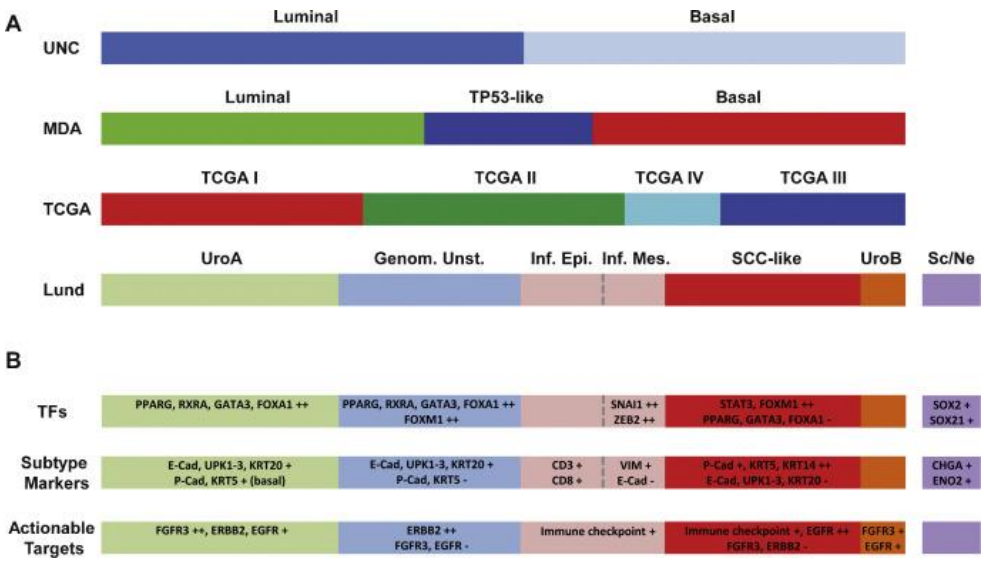


Figure 4. A) Schematic representation of subtype interrelationship, by Aine et al. (2015) [380] using first generation classifiers in 234 tumors of the TCGA dataset. B) Transcriptional regulators, marker genes, and potentially actionable targets.

4.5. Aarhus UROMOL classification of NMIBC

While muscle invasive bladder cancer has been emphasized in recent profiling efforts, Hedegaard et al. performed an extensive molecular characterization of NMI bladder cancer using RNA-seq (n=476) as part of the UROMOL project [182]. A majority of tumors were of stage Ta (nTa=345, nT1=112, nTis=3, nMI=16). Three subgroups were found using gene expression clustering, where the large Class 2 group contained tumors with higher EORTC scores and the vast majority of T1 and MI tumors, while Class 1 and Class 3 mainly contained low grade Ta tumors. Comparison between classification systems indicated that Class 1 was associated with Lund UroA, Class 2 with Lund GU and Infiltrated, and Class 3 with UNC Basal-like. Application of a classifier for the 3 classes in the Lund cohort showed that Class 1 and Class 2 largely separated between low and high stage and grade tumors. Class 1 corresponded with UroA, while Class 2 included GU, BaSq-like, and the high stage and grade UroA, UroB, and a subset of infiltrated tumors. Class 3 was difficult to detect in both the Lund cohort as well as in the TCGA cohort, which could be related to the lower RNA quality of the Class 3 samples or due to technical differences between the studies. Mutation calling from RNA-seq data provided an expanded view of the mutational spectrum in NMI bladder cancer. Summarized, 86% of tumors had some mutation occurring in genes involved in chromatin remodeling, 59% of tumors had mutations in PI3K/AKT/mTOR genes, 52% in DNA damage response genes, and 35% and 20% had mutations in MAPK/ERK or ERBB genes, respectively. Overexpression of late cell cycle genes and APOBEC mutation signature was found to be associated with the more aggressive Class 2 group.

4.6. Second generation of molecular classification of bladder cancer

4.7. UNC and MDA

Since the original publications, the respective classification schemes have each undergone revisions and expanded the number of subgroups. The UNC group analyzed gene expression from 408 tumors of the TCGA dataset, and extended the 2-tiered classification with a “Claudin-low” group [381]. This group largely corresponded to the Basal tumors with the highest expression of immune and stromal signatures. As the majority of gene expression signal in these samples originate from the immune and stromal cells, which does not express epithelial

markers (e.g. claudins), it is questionable whether this group represents an intrinsic subtype. The MDA group analyzed muscle invasive tumors from four cohorts (Lund, TCGA, 2 x MDA). The classification was expanded to five classes, where the p53-like group was split into a Luminal-p53-like and a Basal-p53-like group, representing the most immune and stromal infiltrated tumors of each respective class [382]. IHC analysis of genes that defined the p53-like group confirmed that expression was predominantly originating from non-tumor cells. Notably, a small group of tumors was identified which showed low expression of both Basal and Luminal markers and therefore termed "double-negative". In relation to the Lund taxonomy, the "Claudin-low" corresponds to highly infiltrated BaSq-like tumors and Mesenchymal-like tumors, while the "double negative" corresponds to the Mesenchymal-like and Small-cell/Neuroendocrine-like subtypes.

4.8. TCGA

The second TCGA analysis of bladder cancer was recently published, providing the most comprehensive multi-omic profiling study of MIBC to date (n=408). The previous four cluster classification was refined to include five subtypes using nonnegative matrix factorization (NMF) clustering of global gene expression. The proposed groups included Luminal-papillary (n=142), Luminal-infiltrated (n=78), Luminal (n=26), Basal-Squamous (n=142), and Neuronal (n=20). Distinct tumor categories could also be defined based on mutation signatures, methylation, miRNA, and lncRNAs, as well as protein expression using reverse phase protein arrays. Clustering on both miRNA and lncRNA provided discrete groupings with strong associations to mutations, pathology and expression signatures. A cluster-of-cluster assignment (COCA) analysis showed that tumor groupings from different data types did not fully overlap, and indicates that further sub stratification may be needed. Mutation signature analysis indicated the presence of four discrete mutation categories in the cohort; a small group with the highest overall mutation burden with strong APOBEC signature contribution, a group with frequent *ERCC2* mutations and high ERCC2 mutation signature contribution, a group with intermediate mutation load with predominantly APOBEC signature mutations with low to intermediate mutation load contributions from C>T transition and ERCC2 signature mutations. The largest group (>50% of cohort) showed a lower overall mutation load, with an increased proportions of C>T transition mutations compared to the other groups, and varying degrees of APOBEC and ERCC2 signature mutation patterns. A single hyper-mutated tumor was identified that showed extensive POLE (DNA polymerase epsilon) associated mutations. Better survival was associated with high overall mutation load, high APOBEC mutation load, and high neoantigen load. Clustering of lncRNA resulted

in four clusters, as did clustering on miRNA expression. A notable grouping of node negative *FGFR3* mutated tumors with papillary histology was achieved when lncRNA was used for clustering, suggesting that interrogating the lncRNA expression profiles may be highly informative. Among the mRNA clusters, the three luminal groups showed expression of urothelial markers including *GATA3*, *FOXA1*, UPKs and *FGFR3*. The Luminal-papillary subtype corresponded to the previous Cluster I, and showed enrichment for *FGFR3* mutations and papillary histology. The Luminal-infiltrated and Luminal subtypes corresponded to the previous Cluster II group, where Luminal-infiltrated showed low tumor purity and high expression of stromal genes in addition to urothelial markers. The smaller Luminal subtype showed high expression of UPKs and *KRT20*, and frequently harbored *TP53* mutations. Among the non-luminal tumors, The Basal-Squamous subtype corresponded with Clusters III and IV. The vast majority of tumors in the Basal-Squamous subgroup showed loss of *GATA3* and *FOXA1* expression and high expression of *KRT5* and *KRT14*, showed frequent *TP53* mutations, and contained nearly all cases with reported signs of squamous differentiation. The Neuronal subtype included 3 out of 4 tumors with histological signs of neuroendocrine histology and expressed neuroendocrine markers, corroborating earlier reports of a Small-cell/Neuroendocrine-like subtype [380]. Notably, in both cohorts where this subtype has been identified, not all tumors with this transcription profile showed signs of neuroendocrine histology, and the subtype thus requires mRNA or IHC analysis for detection. This subtype was associated with the overall worst prognosis in the TCGA dataset. In depth analysis should reveal whether these tumors originate from aberrantly differentiated urothelium as would seem likely [383], or if they have a neuroendocrine origin. It is worth exploring whether Etoposide-cisplatin therapy, commonly used in the treatment of bladder cancer with neuroendocrine variant histology, may benefit patients with tumors of this high risk subtype. A strong association between tumor purity and clusters was observed, and the authors conclude that integrative analysis incorporating lncRNAs, miRNAs, and regulatory mechanisms may help further refine molecular subtyping in bladder cancer.

4.9. Lund taxonomy - Global mRNA classification versus tumor - cell phenotype

A limitation of global gene expression analysis of tumor biopsies is that such samples frequently are contaminated with normal cells, such as infiltrating stromal and immunological cells. This is very frequently overlooked, and consequently many of the subtypes defined by gene expression are in fact the result of contaminating cells and not true *tumor cell phenotypes*. To address this, Sjödahl et al. performed careful tissue sampling of a cohort of 307 advanced bladder tumors and performed gene expression profiling paired with extensive tissue microarray IHC analysis of 28 proteins [384]. This revealed a substantial disagreement between groupings obtained through global mRNA clustering and *tumor cell phenotypes* defined by IHC, where tumors with distinct *tumor cell phenotypes* can both converge or diverge into different mRNA gene expression clusters. The converging of tumors with different *tumor cell phenotype* and diverging of tumors with identical *tumor cell phenotype* suggests that broad global commonalities related to the invasive process may exist in muscle invasive tumors. The effect appears partly driven by the degree of immune and stromal infiltration, but also by high proliferation. Each of these aspects can be observed as large cohesive gene expression signatures, which have a high impact on the outcome of hierarchical clustering methods. By utilizing both *tumor cell phenotype* defined by IHC markers and global mRNA cluster assignment, the majority of tumors were classified as Urothelial-like, Genomically Unstable, or BaSq-like. The presence of the minor Small-cell/Neuroendocrine-like (Sc/Ne-like) and Mesenchymal-like (Mes-like) subtypes reported by Aine et al. could be confirmed, each representing approximately 5% of the cohort. The Small-cell/Neuroendocrine-like subtype showed expression of markers indicative of a small-cell neuroendocrine phenotype, including mRNA expression of chromogranin (*CHGA*), synaptophysin (*SYP*), or Neuron-specific enolase (*ENO2*), and protein expression of CHGA, SYP, and NCAM1 (CD56). This subtype also showed increased mRNA levels and tumor cell expression of several tubulins. Tumors of the Mesenchymal-like subtype co-clustered with BaSq-like tumors as both have a high mesenchymal stroma component. Using IHC it was evident that mesenchymal marker expression (e.g. VIM and ZEB2) was limited to the surrounding stromal tissue in BaSq-like tumors, whereas Mes-like tumors displayed expression also by the tumor cells. Similarly, UroB tumors were found to co-cluster with the BaSq-like tumors due to their similarly high expression of keratinization genes. Tumors with Urothelial-like expression patterns were frequent also in this predominantly muscle invasive cohort; however a more notable heterogeneity was observed compared to their NMI counterpart. Urothelial-like tumors that most strongly resembled NMI UroA formed a separate mRNA cluster enriched for lower tumor stage (pT1) and grade

(WHO 1999 G2), however frequent aberrant UPK and KRT20 expression patterns suggests that corruption of the urothelial differentiation program may occur during progression. The UroA subtype in muscle invasive bladder cancer was subsequently termed UroA-Prog – a recognition that although they share much of the UroA molecular profile, there are strong differences between an UroA tumor of low stage/grade and one that has progressed to require radical cystectomy. Similar to observations by Aine et al., a subset of Urothelial-like phenotype classified tumors co-clustered with tumors of the Genomically Unstable subtype. These tumors were almost invariably of high grade, displayed a loss of urothelial-like stratification, and were termed "Urothelial-like C" to reflect their more advanced characteristics. Tumors of the Genomically Unstable *tumor cell phenotype* were also found to co-cluster with the Sc/Ne-like tumors as both groups showed high expression of cell cycle genes and frequent focal amplifications of the *E2F3*/*CDKAL1*/*SOX4* locus at 6p22. A cluster of highly immune and stroma infiltrated tumors was found to contain a mixture of Urothelial-like and Genomically Unstable subtypes when examining at the *tumor cell phenotypes*. The study concluded that particular care must be taken when classifying admixture biopsies such as muscle invasive tumors, and suggests that a bi-nominal classification system that takes both the *tumor cell phenotype* and the gene expression cluster (i.e. context) into consideration is needed.

4.10. Lund taxonomy – validation of a *tumor cell phenotype* informed molecular classification

The work by Sjödahl et al.[384] represents a significant departure from the conventional way that molecular subtyping has been performed, and challenges the objectives for classification and questions what a tumor subtype signifies; a *tumor cell phenotype* or the community of cells. To determine whether stratification on *tumor cell phenotypes* better captures intrinsic properties of bladder cancer Marzouka et al. performed a supervised tumor grouping of this cohort [303]. The *tumor cell phenotype* based grouping included UroA-Prog, UroB, UroC, GU, BaSq, Mes-like, and Sc/Ne, as well as their infiltrated counterparts Uro-Inf, GU-Inf 1, GU-Inf 2, BaSq-like-Inf. To validate this approach, an mRNA centroid classifier based on this grouping was generated and applied to the TCGA MIBC cohort (n=407), revealing excellent associations between subtypes and established key mutations, genomic alterations, expression signatures, and transcription factor expression. *FGFR3* mutations were largely confined to the UroA-Prog and UroB subtypes (44% and 50%, respectively) whereas other groups, notably also UroC (4%), were almost devoid of mutations. Homozygous losses of *CDKN2A* were frequent in the Uro subtypes (39%), as well as in the

BaSq-like subtype, but only in 5% of GU tumors. Conversely, *RB1* mutations and losses were frequent in the GU subtype (44%) and in BaSq-like tumors, but very infrequent in the Uro subtypes. UroC and GU tumors both exhibited an enrichment of *RAF1*/*PPARG* copy number gains, as well as 6p22 amplifications. Although UroC displays characteristic genomic features of Uro, the lack of hardwired FGFR3 activation may allow them to progress on a route more similar to that of GU tumors. The BaSq-like group has repeatedly been identified in studies by different groups, however the diverse mutations and lack of uniquely associated genomic alterations indicates that this group in particularly needs to be further studied, particularly in terms of phenotypic stability and plasticity. Genomically Unstable subtype showed the highest tumor mutation burden and was proposed as a good candidate group for immune checkpoint inhibition therapy. The study also highlighted the poor survival of patients with tumors of the Sc/Ne-like and the UroB subtype in the TCGA cohort.

The multitude of significant associations between subtypes and genomic alterations observed in this study is noteworthy as the classifier was only trained on gene expression data clusters that were reorganized and assembled by IHC determined *tumor cell phenotypes*. This illustrates the strong link between the genomic underpinnings and the tumor phenotype, and strongly connects earlier genomic studies and pathological observations to current mRNA expression based subtyping efforts.

When comparing the Lund 2017 [303] classification with the TCGA 2017 [170] classification (Figure 5) there is high agreement between the Lund Sc/Ne-like and the TCGA Neuronal subtype calls, as well as between BaSq-like calls. The Mesenchymal-like group was not called by the TCGA and is classed as either Luminal-infiltrated or BaSq-like. This group is difficult to confidently classify solely based on global gene expression as its expression profile resembles that of tumors with the highest stromal infiltration. The identification of tumors with a *de facto* Mes-like *tumor cell phenotype* may require IHC analysis or another type of data. The Lund Uro-Inf and GU-Inf groups broadly fall under the TCGA Luminal-infiltrated class, whereas a subset of UroC constitutes those classified as Luminal by the TCGA.

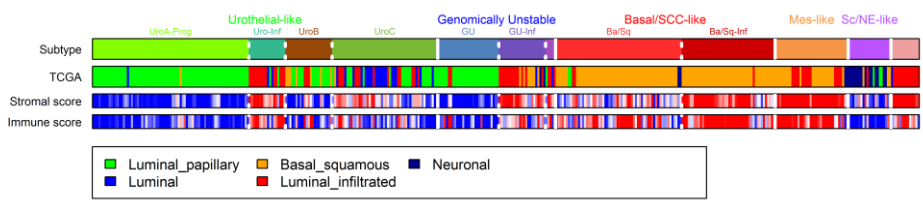


Figure 5. Comparison between Lund 2017 and TCGA 2017 classification.

4.11. Transcriptional regulation of bladder cancer subtypes

The recognized propensity for divergent differentiation in urothelial tumors is reflected in the variety of subtypes that have been described. Corruption of the regulatory pathways of normal urothelial stratification and differentiation appear to lie at the heart of several molecular subtypes. PPARG, FOXA1, and GATA3 and other key transcription factors involved in the development and differentiation of normal urothelium have repeatedly been shown to be defining factors in the tumor subtypes that retain a degree of normal urothelial differentiation or expression of urothelial markers, whereas loss of these transcription factors are strongly associated with the non-urothelial-like subtypes [170,302,303,374,375,378,382,385,386]. Similarly, retinoic acid (RA) signaling is a crucial component of the development of urothelium, and dysregulations of this signaling have been observed in bladder cancer [387-390]. The BaSq-like subtype showed downregulation of ALDH1A2 and overexpression of CYP26B1, involved in synthesis and degradation of the retinoid ligands, respectively [302]. Both retinoic acid receptors (RAR) and Peroxisome proliferator-activated receptor (PPAR) proteins form heterodimers with retinoid X receptor (RXR) proteins, and the expression pattern of several genes involved ligand shuttling to these nuclear hormone receptor dimers (e.g. FABP4, FABP5, and CRABP2) appears altered between subtypes [302]. In BaSq-like tumors EGFR, STAT3, and ΔNp63 appear to be important driving factors of the observed expression patterns [170,302,378,391]. Several components of the hedgehog signaling show differential expression between subtypes. The interplay between hedgehog proteins (SHH, IHH, and DHH), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), WNTs, GLIs, HOX, and TBF-β signaling is extensively studied in developmental biology, where gradient expression and feedback loops are critical for organ formation. When analyzing components of these pathways one cannot rely solely on gene expression of a tumor biopsy, as the spatial organization of signaling gradients and interaction within the stratified urothelium and the stroma is crucial and must be considered. Each regulatory component listed in this section have been extensively studied in both normal and cancer settings, however what is lacking is a comprehensive understanding of how they each contribute to the molecular biology of bladder cancer. In order to accurately interpret the dysregulation that is seen in cancer, it will be paramount that knowledge of the normal developmental biology of the bladder is incorporated in future studies [390,392,393].

4.12. Future perspectives for molecular classification of bladder cancer

Although several different classification systems are currently being used by different groups, they each capture core aspects of the bladder cancer biology. Both clinical trials and retrospective and prospective studies are now frequently utilizing RNA and DNA sequencing techniques and applying molecular classification systems to gain new insights. As the various molecular classifiers and methods mature, reanalysis of this wealth of generated data will undoubtedly provide a vastly better understanding of bladder cancer and how to treat it. It should be kept in mind that the current classification systems are still developing. Key areas that demands further work include; classification methodology, accounting for the tumor microenvironment, integrating multi-level data, and providing clinical value.

4.13. Tumor microenvironment

Tumor classification efforts will undoubtedly need to integrate high precision assaying of the heterogeneous cellular composition of the tumor microenvironment. A descriptor of the tumor composition will likely be crucial for predicting immune checkpoint therapy response, but will also help elucidate how non-tumor cells influence the tumor cell phenotype through paracrine signaling mechanisms [394]. Immunohistochemistry will be a powerful tool for identifying both the composition and spatial organization of non-tumor cells, and its value will likely be greatly enhanced through advancements in artificial intelligence powered image recognition software. Single-cell RNA sequencing (scRNA-Seq) enables transcriptomic analysis of the different cell populations in a tumor, and is one of the most powerful methods available for uncovering the role that stromal, immune and endothelial cells have in tumor initiation, progression and treatment resistance. It is also likely that microenvironment gene expression signatures themselves have prognostic values independent of the intrinsic tumor subtype. The methodological and bioinformatic efforts required for the analysis of such data currently limits its direct applicability in extended profiling studies. Nevertheless, results from scRNA-Seq studies are likely to become incorporated into subtyping efforts through *in silico* deconvolution of bulk tumor expression profiles. While the expression profiles of individual tumor cells should be expected to vary significantly due to tumor heterogeneity, the expression profiles of non-tumor cells may be comparatively stable. Deconvolution of bulk tumor gene expression is a challenging computational task, but tools such as CIBERSORT have been shown

to generate highly significant estimates of tumor cell compositions [395,396]. These types of deconvolution methods will continue to improve as more scRNA-Seq studies provide more detailed cell type-specific reference gene expression profiles.

4.14. Classification methodology

The most common strategy that gene expression studies have utilized is to generate data for a set of samples and performing some variation of hierarchical clustering on log2 transformed gene expression. Clusters derived from this approach are then used to derive gene signatures that are representative of the clusters, often in the form of a centroid or signature. When a new dataset is to be classified, some variation of similarity measurement (e.g. correlation or Euclidean distance) is used to determine which original class the new samples most resemble. As these classification signatures are based on relative gene expression levels, they are sensitive to cohort composition, preprocessing methodologies, batch effects, and technical variation. They are also heavily impacted by the stromal and immune composition of a tumor biopsy. As such, this type of methodology is not acceptable in a clinical situation where samples come in on a regular basis and the clinic expect an answer as quick as possible to help with treatment decisions. They are also unsuitable in studies with very small or homogeneous cohorts. For this reason, cohort dependent classification systems may have to be transformed into so called "single sample" classifiers, that classify samples independent of any other samples and therefore are "absolute". The Lund group has used subtype defining gene ratios and IHC as a way to overcome some of these problems, but a more robust method is still needed. Paquet et al. demonstrated the alarming effect that the cohort composition had on conventional gene expression classifiers in a large breast cancer dataset (n=4924), and proposed a rule based single sample classifier [397,398]. Their "Absolute Intrinsic Molecular Subtyping" (AIMS) method used sets of binary gene expression level rules (e.g. GeneA>GeneB indicates subtype X, while GeneC>GeneD indicates subtype Y) which gave results that vastly agreed with the PAM50 subtypes, and had the major benefit of being fully stable and cohort insensitive. Seiler et al. have published the first proposed single sample classifier (Genomic Subtyping Classifier, GSC) which use a generalized linear model for classifying claudin-low, basal, luminal-infiltrated, and luminal tumors [399]. A fully realized single sample classifier could substantially improve the ability to compare results between different studies, and several groups are working on developing classifiers of this type.

4.15. Data Integration

The link between mutation load and checkpoint inhibitor response, as well as the association between mutations in DNA damage repair genes and response to checkpoint inhibitor and chemotherapy response all highlights the importance of analyzing not only gene expression patterns. As seen in the publication of Hedegaard et al., an efficient, albeit technically challenging, method that could be applied is mutation calling from RNA-sequencing data [182]. A classification system with strong predictive value for neoadjuvant chemotherapy response likely needs to incorporate some form of mutation analysis in addition to molecular gene expression subtyping, as illustrated by the work of Teo et al., van Allen et al., and Liu et al. [347,349,351]. In regards to targeted therapies, a similar approach may be warranted both for ERBB2, FGFR3, and PIK3K/AKT/mTOR targeted therapy [317,318,330,336,400], although the strong association between subtypes and genomic alteration or overexpression of candidate drug targets (e.g. FGFR3 or EGFR) shows that subtyping alone can be a powerful method for patient stratification.

4.16. Clinical value

Ultimately the value of any molecular classifier lies in its ability to improve patient care. The currently available classification systems have all demonstrated an association between subtypes and survival outcome [66,182,303,374,377,378,381,382,399]. Overall, subtypes that fall on the Luminal side of the spectra tend to be associated with better outcome, however further sub stratification should be taken into account as illustrated by the improved survival rate of UroA-Prog/UroC compared to Genomically Unstable and the poor prognosis UroB subtype of the Lund taxonomy, as well as the survival difference between Luminal-papillary and the Luminal-infiltrated/Luminal subtypes of the TCGA taxonomy. The survival difference between subtypes and the associations with published biomarkers reported by Patschan et al. in a cohort of T1 staged tumors suggests that subtyping can likely aid in risk stratification also in this clinically challenging tumor category [66]. Results from Seiler et al. have indicated that the Basal subtype could be an identifier of patients that have a benefit of neoadjuvant chemotherapy [399]. It should be noted that pathological response was associated with better overall survival in the non-basal groups, as would be expected [401]. The Basal group showed a better overall survival than would be expected based on previous studies, regardless of pathologic response, suggesting that further study in a more controlled setting is needed. The potential benefit of NAC often needs to be weighed against NAC-related toxicity and delay of cystectomy, thus a tool for identifying patients that are unlikely to derive benefit from the treatment and

instead receive early cystectomy is needed. Choi et al. reported that tumors classified as Basal and Luminal had similar NAC response rates, whereas p53-like classified tumors showed a markedly lower response rate [378]. Rebouissou et al. explored targeted treatment against EGFR and found that cell lines and BBN-induced (N-butyl-N-(4-hydroxybutyl)nitrosamine) tumors in mice, with a BaSq-like phenotype, showed growth inhibition when treated with Erlotinib [402]. It should be noted that several putative targets such as EGFR are overexpressed in subtypes without obvious hardwired genomic alterations [317].

The major advantage of molecular subtyping is that it provides a biological and molecular context to which clinical results and discoveries can be anchored. A biologically coherent classification system aids the interpretation of clinical studies and can reveal new therapeutic strategies. This was elegantly demonstrated by Mariathasan et al. who used molecular profiling to elucidate response determinants of Atezolizumab in the Imvigor210 bladder cancer cohort [164]. The study found a multifactorial basis of response to immunotherapy, where mutation/neoantigen load, IFNγ expression, $CD8^+T_{eff}$ signature expression, and TGFβ signaling were associated with response rates. Analysis of the microenvironment composition using IHC showed that spatial immune cell localization patterns (immune desert, immune excluded, or immune inflamed) were associated with both response rate and $CD8^+T_{eff}$ signature expression. Stratification by the Lund taxonomy revealed a significantly higher response rate in Genomically Unstable tumors compared to other subtypes or TCGA classes. Although the GU subtype showed low $CD8^+T_{eff}$ signature expression they uniformly displayed lower levels of TGFβ signaling. The role of TGFβ signaling was further examined by testing anti-PD-L1 and anti-TGFβ antibodies alone or in combination in mouse models with immune excluded tumor types. In these models, either antibody alone showed little to no effect, while combined PD-L1 and TGFβ inhibition resulted in significant reductions in tumor burden and led to increased $CD8^+T_{eff}$ infiltration into the tumor. This study should be seen as an important example on how molecular data from clinical trials can be analyzed, and how deeper insights can be derived by taking genomic alterations, as well as tumor microenvironment and tumor subtype into account.

Although genome-wide molecular profiling techniques are laborious and still relatively costly, we are at the juncture of clinical translation. Profiling efforts, historically confined to academic research, have greatly increasing our understanding of the multi-layered complexity across human cancers [403]. The increasing utilization of comprehensive approaches such as whole-genome, whole-exome, and whole-transcriptome sequencing that is now seen also in the clinical trial setting will provide an exceptional opportunity for novel discoveries, and will help clarify the link between tumor biology, patient outcome, and treatment response. Collaborations and data sharing will be crucial in the effort to determine which molecular features and tumor groupings are clinically relevant. Implementation of genome-wide methods into a routine clinical setting remains challenging due to the required infrastructure, but an increased comprehension on the genomic and transcriptomic scale will facilitate the development of pragmatic methods suitable for clinical application.

5. Aims of the thesis

The work presented in this thesis is a part of the Lund Bladder Cancer Group's ongoing effort to provide a both biologically sound and clinically relevant molecular stratification of bladder cancer. The Lund taxonomy has gradually evolved through an extensive series of genomic, transcriptomic, pathological, and epigenetic studies, with a strong emphasis on describing the core biology of bladder cancer.

The specific aims of the papers in this thesis were:

I. To provide a detailed genomic analysis of recurring alterations with yet unresolved target genes.

II. To characterize the gene regulatory systems that governs bladder cancer subtypes.

III. To evaluate the amplification frequency and molecular context of genes of the epidermal growth factor receptor family in bladder cancer.

IV. To explore the Lund taxonomy in muscle invasive bladder cancer and to compare mRNA based classification to intrinsic tumor cell phenotypes.

V. To develop and validate a tumor cell phenotype informed molecular classification system.

5.1. **Paper I –** Detailed Analysis of Focal Chromosome Arm 1q and 6p Amplifications in Urothelial Carcinoma Reveals Complex Genomic Events on 1q, and SOX4 as a Possible Auxiliary Target on 6p

Introduction

Focal amplifications at 6p22.3 and 1q21–24 are frequent in urothelial carcinomas; however the specific target genes remain unclear. Focal amplifications of 6p22.3 frequently include the transcription factor *E2F3*, which is thought to be the primary amplification target. Reports of amplifications that do not include *E2F3* suggest that the neighboring genes *CDKAL1* and *SOX4* could also be potential target genes. 1q arm amplifications are common in non-muscle invasive bladder cancers, whereas higher stage and grade tumors frequently show complex amplification events occurring at 1q21–24. Due to complexity of the rearrangements it has been difficult to identify a specific 1q21–24 amplification target. The aim of this study was to clarify the amplification targets at these two loci. Based on low resolution BAC array-CGH and methylation derived copy number data for 261 urothelial carcinomas we preselected tumors with amplification events at 1q and 6p and analyzed them using high resolution array-CGH. The genomic analysis was paired with gene expression and sequence analysis.

Results

The 6p22.3 region contains a small set of genes that are frequently co-amplified. The high resolution array-CGH confirmed that all focal amplification events covered the *SOX4* gene, while several amplifications excluded the proposed target gene *E2F3*. The expression of *SOX4* but not *E2F3* was strongly increased in these tumors. When amplified, both *E2F3* and *SOX4* showed increased expression. While E2F3 has a well-studied role in cell cycle regulation, it remains difficult to ascribe an exact role to *SOX4* amplifications in bladder cancer due to its diverse biological functions [404,405]. Our results highlighted that the complex and seemingly random alterations at 1q21-24 targets three separate regions. Each region was amplified both together and as separate segments in several tumors. The refined mapping narrows down the list of putative target genes in this region. In these gene-dense regions, both anti-apoptotic genes (*MCL1*) and chromatin modifiers (*SETDB1* and *CHD1L*) were frequently amplified.

5.2. **Paper II –** Molecular subtypes of urothelial carcinoma are defined by specific gene regulatory systems

Introduction

Gene expression profiling of bladder cancer have revealed that coherent, biologically distinct, gene expression signatures are differentially expressed between subtypes. By exploring the transcriptional regulation of these signatures this study aimed to provide a better understanding of the bladder cancer subtypes. A large database of transcription factor (TF) binding sites from chromatin immunoprecipitation sequencing (ChIP-Seq) was assembled from public datasets. By assigning genes the TF binding sites genome-wide, we created a tool for performing *in silico* TF-binding enrichment analysis. Immunohistochemistry, gene ontology analysis, transcription factor motif analysis, and literature based knowledge databases were used to strengthen the results of the ChIP-Seq enrichment analysis.

Results

Bladder cancer gene expression signatures were obtained by performing quality threshold clustering of the Lund dataset of 308 urothelial carcinomas. In addition, subtype specific expression signatures were obtained by SAM (Significance Analysis of Microarrays). The most distinct pattern was downregulation of genes linked to differentiation in the BaSq-like subtype. Enrichment analysis indicated that GATA3, FOXA1, RXRA, and PPARG are key regulators of this gene cluster. These genes were themselves found within the signature, suggesting that the loss of expression of these factors may be part of the loss of differentiation. A gene signature with strong overexpression in the BaSq-like subtype was linked with keratinization, and regulated by STAT3 and AP-1, which can be activated by RTKs such as EGFR. While overexpression of *EGFR* is a distinct feature of the BaSq-like subtype the mRNA levels of *STAT3* were similar across the subtypes. However, STAT3 and pSTAT3 protein levels were found to be elevated as measured by IHC. A late cell cycle signature was markedly higher in tumors of the UroB, GU, and BaSq-like subtypes compared to UroA, and showed strong enrichment for FOXM1 and factors of the Myb-MuvB cell cycle regulatory complex. A distinct subtype separation by HOX gene expression pattern was further studied. Tumors of the UroA subtype show a pattern of anterior HOXA (HOXA1-HOXA7) and HOXB gene expression. The expression of these genes appears to decrease in the more poorly differentiated higher stage and grade tumors which instead overexpress the posterior HOXA gene (HOXA9-13). The anterior HOXA and HOXB genes contained binding sites for urothelial transcription factors and are also known to be regulated by retinoic acid signaling.

A search for genes with an expression profile reflecting this switch was enriched for genes involved in urothelial differentiation. These genes in turn showed signs of HOX regulation. A comparison with breast cancer indicated a distinct similarity between the regulatory systems of the bladder BaSq-like and the breast Basal-like subtypes, with loss of FOXA1 and GATA3 observed in both, while differing in their respective loss of nuclear hormone receptor (PPARG in bladder and ESR1 in breast). In breast cancer, the HOX gene expression pattern did not show an apparent subtype association.

5.3. **Paper III –** HER2 and EGFR amplification and expression in urothelial carcinoma occurs in distinct biological and molecular contexts

Introduction

Targeted therapies against members of the epidermal growth factor receptor family have shown clinical benefits in other tumor types, e.g. HER2-directed antibodies in breast cancer or tyrosine kinase inhibitors in lung cancer. Despite proven clinical benefits in other malignancies, the handful of clinical trials targeting EGFR and HER2 in bladder cancer has only shown limited efficacy (See section 3.8). We hypothesize that this may partly be due to poor patient stratification. Overexpression of EGFR and HER2 (ERBB2) is common in bladder cancer. From the Lund taxonomy it is clear that overexpression is linked to the described molecular subtypes. While tumors of the Genomically Unstable subtype have strong HER2 expression, EGFR is predominantly overexpressed in the BaSq-like subtype. To explore the relationship between molecular subtypes and epidermal growth factor receptors in detail we examined the Lund 2012 cohort of mixed stage tumors using copy number data, immunohistochemistry, and gene expression analysis. Additionally, we examined the HER2-status using silver *in situ* hybridization (SISH) and IHC following the clinical praxis used for scoring HER2 in breast cancer. Gene expression analysis, immunohistochemistry, SISH staining, and hotspot mutation sequencing was also performed on the Lund 2017 cohort of 400 advanced bladder tumors.

Results

The data revealed that over 20% of tumors had copy number gains or focal amplification of HER2, and that alterations increased both mRNA and protein levels. In non-muscle invasive tumors, amplifications were highly enriched in the Genomically Unstable subtype (45%), but rare in Urothelial-like A (11%) tumors. In muscle invasive tumors, HER2 amplifications and overexpression was frequent in both the Genomically Unstable (47%) and Urothelial-like (38%) subtypes, which suggests that the acquisition of HER2 alterations in NMI UroA may be involved in the progression to muscle invasive disease, and supports earlier reports that HER2 altered NMI bladder cancer have a more aggressive behavior. In contrast, the high frequency of HER2 alterations and overexpression in both NMI and MI Genomically Unstable tumors suggests that this may be a founding feature of this subtype. Although muscle invasive Urothelial-like and Genomically Unstable tumors were frequently HER2-positive, these subtypes have a distinctly different mutation and genomic alteration background which may impact the clinical therapeutic efficacy when targeting HER2. Overexpression of EGFR was predominantly confined to tumors of the BaSq-like subtype, but unlike HER2, overexpression was not strongly associated with genomic amplifications. Strong overexpression of both ERBB2 and EFGR very rarely occurred in the same tumor. Overall, the results show that epidermal growth factor receptor signaling occurs in different molecular contexts and that proper tumor stratification may be needed when evaluating clinical trials.

5.4. **Paper IV –** Molecular classification of urothelial carcinoma: global mRNA classification versus tumour - cell phenotype classification

Introduction

Global mRNA expression profiling is a powerful method for characterizing tumors, and has been used to define molecular subtypes in many forms of cancer. Tumor biopsies contain an admixture of both tumor and non-tumor cells, and the composition strongly impacts gene expression analysis. The effect of non-tumor cells has not been adequately accounted for in previous profiling studies of bladder cancer, and raises the question of whether a subtype label should denote a specific tumor cell type, or a tumor type with a given composite organization. To explore this, a large cohort of advanced bladder cancers was analyzed by gene expression profiling of the composite tumor tissue and through pathological examination of the tumor cells using immunohistochemistry with antibodies for 28 proteins. Careful tissue sampling ensured that representative tissue was obtained for both mRNA extractions and IHC staining.

Results

The previously established Lund subtypes could be identified at both the mRNA and protein level, as could the two proposed minor subtypes Small-cell/Neuroendocrine and Mesenchymal-like. Mesenchymal-like tumors cells expressed EMT markers, whereas the expression of these markers was limited to the stromal tissue in other tumors. The Small-cell/Neuroendocrine subtype expressed neuroendocrine markers, but did not consistently show signs of a variant histology. Hierarchical clustering of gene expression revealed that tumors with the same IHC defined *tumor cell phenotype* could separate into different clusters, and conversely, that tumors with different *tumor cell phenotypes* could co-cluster. This diverging and converging of *tumor cell phenotypes* on the mRNA cluster level was to a large extent driven by the degree of immune and stromal infiltration, but also by shared features of advanced tumors, such as high expression of proliferation associated cell cycle genes. Further analysis revealed that both characteristic gene expression signatures and subtype defining IHC markers were in excellent agreement, but that the signal on the mRNA level can be heavily diluted due to high non-tumor cell content. This work suggests that both *de facto* tumor cell phenotypes and the tumor composition must be taken into account when performing subtype classification. See section 4.9 for a further description of this work.

5.5. **Paper V –** A validation and extended description of the Lund taxonomy for urothelial carcinoma using the TCGA cohort

Introduction

The work in Paper IV highlights both the extensive complexity of muscle invasive tumors and the need to account for the tumor microenvironment when performing classification. To determine how a classification system that takes the tumor cell phenotype into account performs, we created a new mRNA based classifier based on a manually curated tumor grouping that was informed by the IHC phenotypes. The hypothesis was that such a classifier could potentially identify tumor cell phenotypes both in a low infiltration and a high infiltration context, and thus partially circumvent the need for IHC analysis. We applied this classifier to the TCGA gene expression dataset of 408 muscle invasive bladder cancers. This dataset also contains extensive additional data such as mutations and copy number alterations, which was examined for association with the subtype classification.

Results

The *tumor cell phenotype* informed classifier included the subgroups UroA-Prog, UroB, UroC, GU, BaSq, Mes-like, and Sc/Ne, as well as the infiltrated counterparts Uro-Inf, GU-Inf 1, GU-Inf 2, BaSq-like-Inf. The subtypes showed an excellent association with established key mutations, genomic alterations, expression signatures, and transcription factor expression (See section 4.10). Distinct differences in genomic alterations were observed between the different Urothelial-like subtypes, confirming that significant heterogeneity exists among tumors that retain traces of urothelial-like differentiation. Both alteration and expression patterns of the Genomically Unstable subtype corroborated earlier descriptions of this subtype, including *TP53* mutations and *RB1* losses. This subtype also showed the highest overall mutation burden, which may indicate that the tumors of this subtype are more likely to respond to immune checkpoint inhibition therapy. The Urothelial-like B and the Small-cell/Neuroendocrine-like subtype were found to have to worst overall survival. The strong stratification of genomic events obtained by our mRNA based classifier indicates the validity of this tumor stratification approach, and suggests that it may be of value in future studies.

Summary in Swedish

I Sverige drabbas drygt 3 000 personer av urinblåsecancer varje år. Under 2016 insjuknade 2 276 män och 880 kvinnor i blåscancer, vilket gör det till den fjärde vanligaste cancertypen hos män och den åttonde vanligaste hos kvinnor. Både godartade och elakartade blåscancertumörer är problematiska för både patienten och för sjukvården. Även om godartade tumörer generellt sett har en god prognos så tenderar dessa tumörer att återkomma även efter att tumören till synes opererats bort. Detta gör att de drabbade behöver gå på upprepade undersökningar för att se om nya tumörer uppstått, och huruvida dessa är mer aggressiva. Om tumören är aggressiv kan den växa igenom urinblåsan och sprida sig i närliggande vävnad, och till slut sprida sig vidare i kroppen. Prognosen för patienter med avancerad blåscancer som spridit sig är mycket dålig, men upptäcks en aggressiv tumör i ett relativt tidigt skede kan man med radikal kirurgi operera bort hela urinblåsan med en förhoppning att få bort all tumörvävnad. Kirurgin kombineras ofta med cellgiftsbehandling för att avdöda cancerceller som redan spridit sig i kroppen. Trots att blåscancer är så pass vanligt har det historiskt sett bedrivits väldigt lite forskning kring denna cancerform. Detta återspeglas i det faktum att både behandlingsförfarande och prognos länge varit oförändrade.

Blåscancer har under de senaste fem åren studerats intensivt med olika molekylära metoder, vilket har ökat vår förståelse kring sjukdomen markant. Utvecklandet av läkemedel som aktiverar kroppens immunförvar och får det att angripa och bekämpa cancerceller har inneburit ett stort genombrott inom cancerbehandling. Flera så kallade riktade läkemedel är också under utveckling. Dessa baseras på cancercellernas biologi och verkar mycket specifikt mot ett visst protein eller en viss molekyl. Dessa läkemedel har en mycket god verkan i vissa patienter, medan effekten helt uteblir för andra. De etablerade metoderna för att bedöma blåscancer och förutspå sjukdomsförloppet är för tillfället otillräckliga. Detta gäller även huruvida olika behandlingar kommer att vara effektiva eller inte. Den ökade molekylärbiologiska kunskapen kring sjukdomen kan förbättra den nuvarande diagnostiken och hjälpa till att förklara varför olika tumörer är mer godartade än andra, samt varför behandlingar enbart fungerar i vissa patienter.

Vi har visat att man kan dela in blåscancertumörer i flera undergrupper, eller ”subtyper”, baserat på deras molekylära egenskaper. Genom att gruppera tumörer i subtyper får vi en mer överskådlig och tolkningsbar bild över de molekylära mekanismer som orsakar blåscancer. Målet med mitt avhandlingsarbete har varit att kartlägga olika molekylära och biologiska egenskaper hos dessa subtyper.

I första delarbetet undersöker vi två av de vanligaste kromosomförändringarna i blåscancer, amplifiering av korta armen på kromosom 6 och av den långa armen på kromosom 1. Genomiska amplifieringar verkar drivande på tumörväxt, då ett ökat antal genkopior förstärker genuttrycket av s.k. onkgener. Vårt mål var att identifiera potentiella onkogener genom detaljerad kartläggning av dessa förändringar.

I det andra arbetet analyserar vi genuttryck och genamplifiering av generna *EGFR* och *HER2*. Det finns redan cancerläkemedel riktade mot dessa gener med god klinisk verkan i vissa typer av bröst- och lungcancer. När dessa läkemedel testats i blåscancer har de dock visat sig vara ineffektiva i de flesta patienter. Detta tyder på att vi behöver ett sätt att identifiera de patienter där dessa typer av behandlingar har störst chans att fungera. Vi behöver även förstå mekanismerna som avgör om behandlingen är verksam eller inte. Vi kunde visa att *HER2* hade förhöjt uttryck och genamplifiering i två olika subtyper, och uttrycks därmed i två skilda genetiska sammanhang. Detta kan vara en viktig förklaring till varför terapier riktade mot HER2 uppvisar sämre effekt i blåscancer än i andra cancertyper. Kraftigt uttryck av *EGFR* definierar en subtyp av blåscancer som uppvisar tydliga likheter med skivepitelcancer, men vi kunde visa att detta inte var orsakat av genamplifiering. Detta kan vara av stor betydelse för möjligheterna att använda sig av riktade behandlingar mot denna gen.

I det tredje arbetet använde vi en bioinformatisk metod för att identifiera transkriptionsfaktorer som styr de mönster av genuttryck som karakteriserar de skilda undergrupperna av blåscancer. Vi kunde visa att viktiga differentieringsprocesser är helt blockerade i vissa subtyper av blåscancer, en förklaring till varför celler i urinblåsan börjar tappa sitt normala utseende och beteende, och i vissa fall även börja likna helt andra celltyper. Vi kunde även identifiera faktorer som styr biologiska processer som visar ett karakteristiskt påslag i olika blåstumörer. Vi kunde påvisa att flera av de genreglerande system som verkar i blåscancer även verkar i bröst och lungcancer, och därmed kan betraktas som generella.

I det fjärde arbetet undersökte vi en stor serie av aggressiva tumörer både avseende gen- och proteinuttryck. I detta arbetet vidareutvecklade och validerade vi den molekylära klassningen för blåscancer som vår grupp lanserat. Resultaten visade att det finns diskrepanser mellan gruppering på RNA-nivå och på protein-nivå. Denna insikt är av avgörande betydelse både för den biologiska förståelsen och den kliniska hanteringen av blåscancer. En viktig slutsats av vårt resultat är att de molekylära klassningsystem som används i forskningsstudier kan förbättras genom att utnyttja båda dessa typer av information.

I det femte arbetet vidareutvecklade vi vår klassningsmetod baserat på resultaten i det föregående arbetet. Vi validerade vår molekylära klassning genom att applicera den på ett stort dataset som förutom information om genuttryck även hade uttömmande information om genmutationer och kromosomförändringar. Vårt klassningsystem, som gavs namnet ”The Lund Bladder Cancer Molecular Taxonomy”, kunde effektivt identifiera undergrupper av tumörer som uppvisade tydligt skilda mönster av genuttryck, mutationer, och kromosomförändringar.

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