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Precision Molecular Pathology of Bladder Cancer

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Precision Molecular Pathology of Bladder Cancer

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Part I
Molecular Pathology of Bladder Cancer: Current Understanding and Application

Chapter 1
Risk Factors and Molecular Features Associated with Bladder Cancer Development

Anirban P. Mitra, Georg Bartsch Jr., and Richard J. Cote

Introduction

The management of bladder cancer has witnessed several advances in the past decade. Improvements in imaging and visualization technologies now allow for more detailed localization of tumors within the bladder [1, 2], and novel urine-based cancer detection methods offer the opportunity for precise and noninvasive surveillance [3, 4]. Administration of neoadjuvant chemotherapy has demonstrated oncologic benefit [5], and improved peri-operative management protocols have enhanced post-surgical recovery [6]. Despite these developments, however, survival for patients undergoing radical surgery for bladder cancer has remained fairly unchanged over the last 30 years [7]. Indeed, cancer of the urinary bladder remains the sixth-most common malignancy in the United States and the eighth-most frequent cause of cancer-related deaths [8]. Worldwide, the disease accounts for nearly 430,000 new cases and over 165,000 deaths each year [9, 10].

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A variety of factors have been associated with an increased risk of developing bladder cancer, and several of these have been shown to modulate alterations in intracellular factors that deregulate the homeostasis of urothelial cells. The development and progression of urothelial carcinoma (UCa) of the bladder is now known to involve alterations in several molecular pathways. These alterations often dictate the rate of tumor progression, and may therefore act as surrogates for identifying patients who have more aggressive disease. Identifying patient subpopulations based on the molecular alterations in their primary tumors may therefore permit risk stratification and administration of more personalized therapies.

Several factors have been implicated with increased risk of developing bladder cancer, although a direct link with causation has been identified in only a limited number of instances.

Intrinsic Factors

While female gender has a 3–4 times lower incidence rate when compared to men [8], women diagnosed with UCa of the bladder are more likely to have a locally advanced tumor at the time of diagnosis [11]. Furthermore, females are at higher risk of disease recurrence, progression, and mortality after tumor treatment [12, 13]. Many hypotheses have been established to explain this difference in UCa incidence including disparate exposures [14, 15] to bladder cancer risk factors to different sex steroid hormone regulations [16–18] and differing metabolic detoxification of carcinogens [19, 20].

Few data have been published on the impact of race as risk factor for UCa. A Surveillance, Epidemiology and End Results analysis identified African American race to be associated with adverse stage at initial presentation and reduced survival rates [21]. In the same analysis, African Americans were shown to have a lower age-standardized incidence rate. Marital status has been associated with a better UCa survival, although no associations have been made with risk for bladder cancer. Survival rate for married men are more favorable when compared to unmarried, and this was an independent factor despite race comorbidities, and other socioeconomic factors [22].

Lifestyle-Associated Factors

Tobacco smoking is the most important risk for UCa and is associated with approximately 50% of tumors (former tobacco smoking—hazard ratio, HR: 2.22; 95% CI, 2.03–2.44; current tobacco smoking—HR 4.1; 95% CI, 3.7–4.5) [23]. Aromatic amines including ß-naphthylamine and polycyclic aromatic amines in tobacco cause UCa. The latency period for UCa is over 30 years. Inhalation of tobacco smoke into the lungs is associated with greater risk compared with inhaling into the mouth only [23, 24]. There is a difference in risk of UCa between patients smoking black and

blond tobacco. Black tobacco in former times exceeded the amount of *N*-nitrosamine and 2-naphylamine [24]. Environmental tobacco smoke exposure was found to be significantly associated with a higher rate of UCa of the bladder when exposed to cigarette smoke in childhood and adulthood (HR 3.08 95% CI, 1.16–8.22) [25].

In a meta-analysis that included 27,784 subjects, high levels of physical activity were associated with decreased UCa risk (relative risk, RR 0.85, 95% CI, 0.74–0.98; I^2 83%, $p < 0.001$). In cohort studies, similar results were noticeable (RR 0.89; 95% CI, 0.80–1.00; I^2 64%). These results were comparable for female (RR 0.83; 95% CI, 0.73–0.94; I^2 0%) and male gender (RR 0.92; 95% CI, 0.82-1.05; I^2 77%, *p* value for difference = 0.657). No significant difference was shown between recreational (RR 0.81, 95% CI, 0.66–0.99; I^2 77%) occupational physical activity (RR 0.90; 95% CI, 0.76–1.00; I^2 76%, p value for difference = 0.374) [26].

A meta-analysis investigated the personal use of hair dyes and UCa risk using 15 case-control and two cohort studies (8504 cases/deaths, 14,102 controls and 617,937 persons at risk). When compared with no hair dye use, the pooled RR of UCa of the bladder with any hair dye use was 0.93 (95% CI, 0.82, −1.05) with moderate heterogeneity among studies (I^2 34.1%, $p = 0.07$). Similar RRs were found for females (RR 0.95) and males (RR 0.81). Based on seven studies, the pooled RR for the use of permanent hair dyes was 0.92 (95% CI, 0.77–1.09). For the use of dark-colored dyes, the RR was 1.29 (95% CI, 0.98–1.71). The study concluded that personal hair dye users are not at significant higher risk of developing UCa [27].

Diet

Data on the relationship of total fluid intake and bladder UCa risk is controversial. In a case-control study, Michaud et al. found that subjects with a fluid intake >1.4 L/day compared with 0.4 L/day halved their risk of developing UCa (HR: 0.47; 95% CI, 0.33–0.66) [28]. On the other hand, Bai et al. found no statistically significant association between total fluid intake and UCa (RR 1.12; 95% CI, 0.94–1.33; I^2 82.8%; $n = 14$) [29]. Meta-analyses have also not identified a statistically significant association between coffee consumption and UCa risk (RR: 1.12; 95% CI, 0.80–1.44) [30–32]. Another meta-analysis found no statistically significant association for UCa risk among heavy alcohol drinkers (≥3 drinks/≥37.5 g/day; RR: 0.95; 95% CI, 0.89–1.07) [33]. Eating processed meat has been associated with increased risk of UCa (RR 1.22; 95% CI, 1.04–1.43; $n = 11$) but this association has not been noted with red meat (RR 1.15; 95% CI, 0.97–1.36) [34].

In a meta-analysis including six case-control studies and two cohort studies, ever consumption of chlorinated drinking water was associated with an increased risk of bladder UCa in men (combined odds ratio, OR 1.4; 95% CI, 1.1–1.9) and women (combined OR 1.2; 95% CI, 0.7–1.8). OR for mid-term exposure was 1.1 (95% CI 1.0–1.2) and for long-term exposure was 1.4 (95% CI 1.2–1.7) [35]. In a meta-analysis evaluating 28 studies investigating the association between arsenic and drinking water and UCa, the predicted risk were 2.7 (95% CI 1.2–4.1), 4.2

(95% CI 2.1–6.3), and 5.8 (95% CI 2.9–8.7) for arsenic levels of 10, 50, and 150 mg/L in drinking water. Bootstrapped randomizations confirmed this increased risk, but lowering the effect size to 1.4 (95% CI 0.35–4.0), 2.3 (95% CI 0.59–6.4), and 3.1 (95% CI 0.80–8.9). Arsenic in drinking water is associated with an increased risk of UCa although this is uncertain at lower levels (<150 mg/L) [36].

Occupational Exposure

After tobacco smoking, occupational exposures to carcinogens are the most important risk factor for the development of bladder UCa. Approximately 20% of UCa cases are related to exposure to occupational carcinogen exposure [15].

Aromatic amines including 2-naphtylamine, 4-aminobiphenyl, and benzidine are evident in products from the chemical, dye, and rubber industries as well as in hair dyes, paints, fungicides, cigarette smoke, plastics, metal and motor vehicle exhaust and pollutant emissions from industrial instillations [37–40]. In 1954, a 200-fold increase of bladder UCa risk was documented for English and Welsh workers exposed to 2-naphtylamine. The standardized mortality rates in more than 11,000 workers was observed for "storage and shipment" to be 253 (95% CI 93–551) and for "general work" to be 159 (95% CI 92–279) [41]. Benzidine is used in dye and rubber production and has been identified to be the most important carcinogenic aromatic amine causing human bladder damage [42]. 92 out of 331 workers, who had been exposed to benzidine production in a German facility before 1967 eventually contracted bladder cancer [43]. In a Chinese cohort study with 784 workers with benzidine exposure, a 35-fold increase in UCa risk was observed [44]. 4,4′-methylenebis(2-chloroaniline) is a synthetic chemical widely used in castable polyurethane parts. It is either inhaled or absorbed over the skin and increases the risk of developing UCa [39].

Drug and Therapy Exposure

High-level intake of phenacetin-containing analgesics has been associated with increased UCa risk. However, only inconsistent data exists associating paracetamol, its main metabolite, with increased UCa risk [36]. In a large case-control study, the RR of UCa for regular users of nonsteroidal anti-inflammatory drugs was 1.52 (95% CI, 0.9–2.7) for phenacetin and 0.85 (95% CI, 0.6–1.2) for paracetamol [45]. On the other hand, nonsteroidal anti-inflammatory drugs had a protective effect against UCa genesis in animal models [46].

The use of cyclophosphamide has been associated with an increased risk of developing UCa [36]. The antidiabetic drug pioglitazone has been the recent focus of attention regarding its potential association with increased UCa risk. In a recent epidemiological study, the incidences of UCa were 89.8 and 75.9 per 100,000 person-years for pioglitazone users and nonusers, respectively. Further analyses revealed no

increased risk for pioglitazone use to cause UCa (HR 1.06; 95% CI, 0.89–1.26) [47]. However, a meta-analysis of pioglitazone involving 215,142 patients demonstrated an increased risk of developing UCa (HR 1.23; 95% CI, 1.09–1.39) [48]. Further definitive studies regarding the association of pioglitazone and UCa are awaited.

Indwelling urinary catheters have been known to induce histological changes in the bladder with time. This is especially notable in patients with spinal cord injuries, with increased incidence of UCa, squamous cell carcinoma, and adenocarcinoma of the bladder [49]. Incidence of squamous cell carcinoma is more common in patients with indwelling urethral catheters and suprapubic tubes than in those using clean intermittent catheterization or condom catheterization [50]. One retrospective study noted that after controlling for confounding variables, subjects with spinal cord injuries using solely indwelling urinary catheters had a significantly greater risk of bladder cancer (RR 4.9; 95% CI, 1.3–13.8) than those using non-indwelling methods. Additionally, mortality caused by bladder cancer in individuals with spinal cord injuries was significantly greater than that of the US population [51].

Pathology Risk Factors

Infection by *Schistosoma haematobium* leading to cystitis is common in some regions, especially in Northern Africa. Such chronic irritation of the bladder wall increases the risk of bladder cancer, especially that of squamous cell carcinoma of the bladder [52]. The risk of bladder cancer in patients reporting a history of urinary schistosomiasis was 1.72 (95% CI, 1.0–2.9), and was further increased in male smokers (RR 15.8) [53]. Chronic irritation by urinary tract infections and urinary calculi also increase bladder cancer risk, particularly squamous cell carcinoma, as has been demonstrated in several case-control studies [54–56]. Most of the studies showed a twofold increase in risk in development of bladder cancer.

Several studies have also demonstrated an association between human papillomavirus infection and bladder cancer. A study from Egypt that analyzed bladder cancer cases associated with *S. haematobium* noted human papillomavirus DNA detected in 49% of cases, the majority of which were of type 16 [57]. A meta-analysis showed a human papillomavirus prevalence of 16.88% among the bladder cancer cases, most of whom were high-risk types, especially type 16. Patients who detected positive for human papillomavirus were at a significantly increased risk of bladder cancer (OR 2.84; 95% CI, 1.39–5.80) [58].

Hereditary Genetic Alterations

Generally, the risk of bladder UCa is twofold higher in first-degree relatives of UCa patients [15]. Individual susceptibility to extrinsic carcinogens, e.g. for tobacco smoke, has been demonstrated for *N*-acetyl transferase enzymes (NAT1,

NAT2), which are involved in bioactivation and detoxification of such carcinogens [59]. Genetic factors such as slow acetylator NAT2 variants or glutathione *S*-transferase μ1 (GSTM1)-null genotypes have been identified as risk factors for bladder UCa [15]. While both NAT2 slow acetylation and GSTM-1-null genotypes show similar associations among superficial and muscle-invasive UCa, fibroblast growth factor receptor 3 (*FGFR3*) is often mutated in noninvasive low-grade tumors [60, 61]. Three large genome-wide association studies have identified common sequence variants associated with UCa on chromosomes 2q, 3q, 4p, 5p, 8q, 19q, and 22q (e.g., missense variant rs2294008 in the prostate stem cell antigen gene (*PSCA*) HR 1.15, 95% CI 1.10–1.20; and T-allele of rs798766 on 4p16.3–HR 1.24, 95% CI, 1.17–1.32) [61–63]. Another genetic predisposition recently was demonstrated with solute carrier family 14 (urea transporter) gene (*SLC14A*), which is related to renal urine concentration and therefore with variations in contact of carcinogens with urothelial cells (HR 1.17; 95% CI, 1.11–1.22) [19].

Molecular Pathways of Bladder Cancer Development

UCa can present as a noninvasive phenotype where malignant cells are restricted to the urothelial layer, and an invasive phenotype wherein tumor cells breach the basement membrane and may invade the subepithelial connective tissue and underlying muscle [64]. Noninvasive UCa may present in two forms. Papillary (Ta) tumors are exophytic, tend to recur locally, but rarely invade the basement membrane or metastasize. However, carcinoma in situ (CIS) is a flat lesion with a high propensity for invasion and metastasis. Patients with only CIS lesions in their urinary tract may have synchronous and/or develop metachronous tumors [65]. Ta tumors develop due to molecular aberrations that are usually distinct from CIS and invasive (T1–T4) cancers, although these pathways may not be mutually exclusive (Fig. 1.1) [66, 67]. Low-grade papillary tumors usually have a constitutively active receptor tyrosine kinase–Ras pathway, with activating mutations in *HRAS* and *FGFR3* [68–70]. High-grade Ta tumors are often characterized by homozygous deletion of $p16^{INK4a}$ [71]. CIS and invasive tumors frequently have alterations in *TP53* and retinoblastoma (*RB*) genes and pathways [72]. Loss of heterozygosity of chromosome 9q is more frequent in low-grade Ta tumors, although chromosome-9 deletions may be found in both dysplastic urothelium and CIS lesions [73, 74]. When the occasional papillary tumor transforms to an invasive phenotype, this is usually due to accumulation of additional p53-pathway alterations. p16 alterations have also been identified in invasive tumors [75]. Alterations in cadherins, matrix metalloproteinases (MMPs), vascular endothelial growth factors (VEGFs), and thrombospondin-1 (TSP-1), which remodel the extracellular matrix and promote tumor angiogenesis, are more common in muscle-invasive (T2–T4) neoplasms and also contribute to nodal metastasis [66].

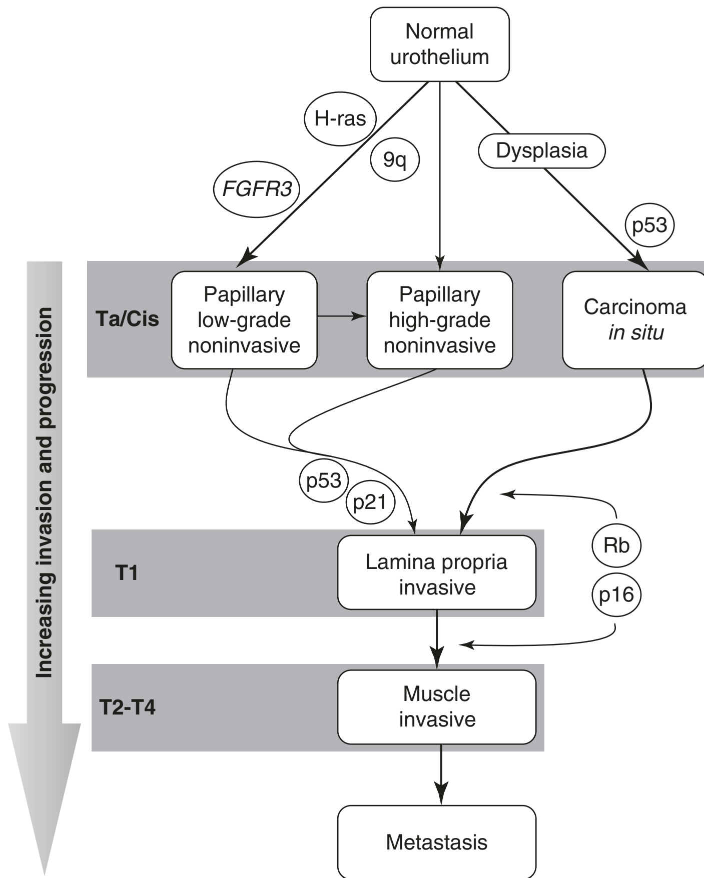

Fig. 1.1 Model for urothelial tumorigenesis and progression. Noninvasive and invasive tumors have unique molecular profiles and arise from distinct pathways. The locations of molecules indicate events that pose a risk for progression of a particular phenotype. The rare papillary carcinomas that invade are more likely to have genetic alterations at crucial loci. Long arrowheads represent higher relative frequency of occurrence. *Abbreviations*: *H-ras* protein of the Harvey rat sarcoma viral oncogene homolog gene, *FGFR3* fibroblast growth factor receptor 3. Adapted from [72]. All rights reserved

In addition to the differences in molecular alterations between noninvasive and invasive UCa, there are also marked contrasts in risk association and chromosomal alterations between these two clinical phenotypes (Fig. 1.2). Smokers have a much higher risk of developing invasive tumors than noninvasive tumors, and this is especially true in current smokers (as opposed to ex-smokers) [76]. Invasive tumors also have more chromosomal aberrations than Ta tumors [77]. In addition, post-cystectomy recurrences are higher in patients with muscle-invasive cancers than those with non-muscle-invasive (superficial) tumors, and prognosis following such recurrence is generally poor [78].

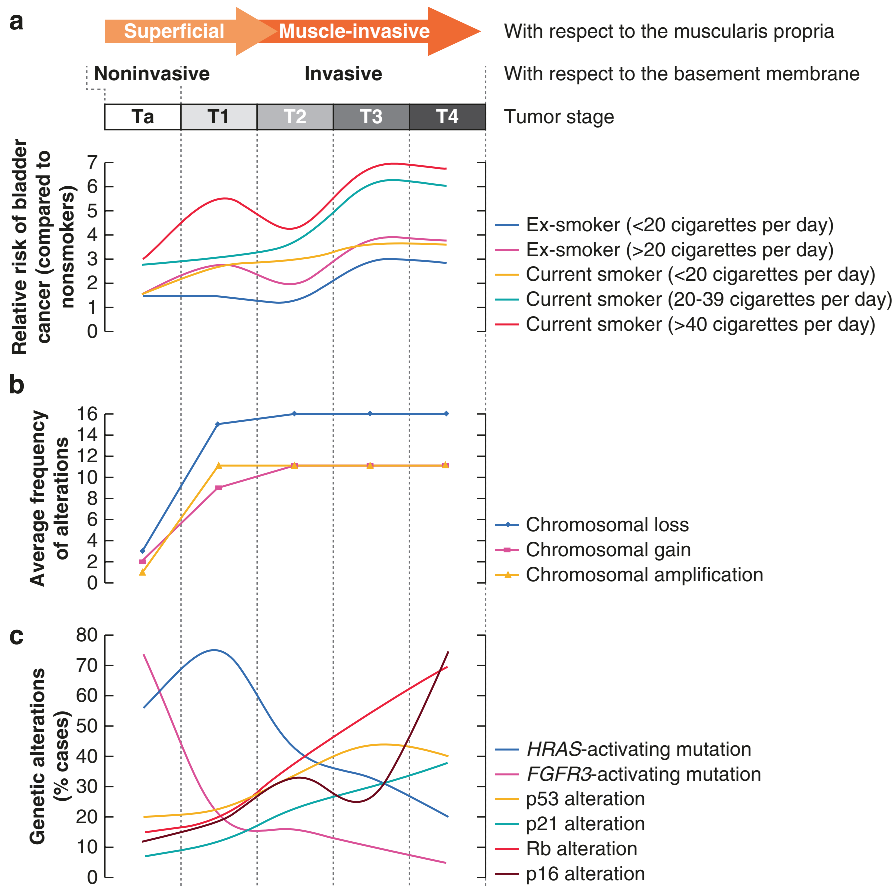

Fig. 1.2 Differences between noninvasive and invasive bladder tumors. Superficial bladder tumors are lesions that do not invade the muscularis propria (Ta, T1). However, noninvasive tumors refer to those that do not invade the basement membrane (Ta). (**a**) The relative risk of developing invasive bladder cancer is higher in smokers than in nonsmokers. (**b**) Invasive tumors also have a higher frequency of chromosomal losses, gains, and amplifications. (**c**) Although mutations in *HRAS* and *FGFR3* decrease with invasion, the opposite is true for p53, p21, Rb, and p16 alterations. *Abbreviations*: *FGFR3* fibroblast growth factor receptor 3 gene, *HRAS* Harvey rat sarcoma viral oncogene homolog gene, *Rb* retinoblastoma protein. Adapted from [80]. Copyright 2009 Annual Reviews. All rights reserved

Molecular Pathways of Bladder Cancer Progression

Bladder tumorigenesis involves alterations in multiple homeostatic pathways with complex deregulations within an intricate molecular circuitry (Fig. 1.3). These deregulations ultimately establish the tumor's fate [79]. Therefore, these alterations often serve as predictors of outcome, and may also act as therapeutic targets [80–82].

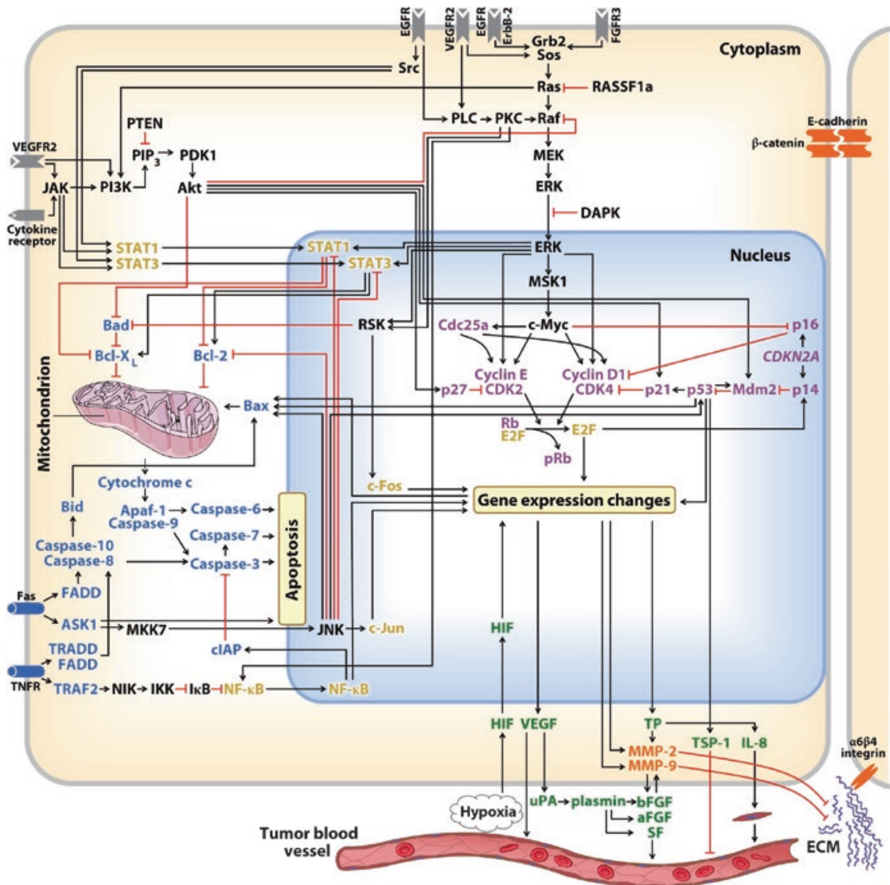

Fig. 1.3 Intra- and intercellular circuitry contributing to bladder tumorigenesis. A complex network of molecular signaling is involved in malignant transformation and tumor progression. Mitogenic signals from growth receptors (*gray*) on the cell surface are conducted along signaling pathways (molecules in *black*) to affect cell-cycle regulation (molecules in *purple*) and apoptosis (molecules in *blue*). This leads to gene-expression changes that are controlled by key transcription factors (*yellow*). The tumor cell also interacts with factors controlling angiogenesis (*green*) and invasion (*orange*). *Abbreviations*: *aFGF* acidic fibroblast growth factor, *ASK1* activator of S phase kinase 1, *bFGF* basic fibroblast growth factor, *DAPK* death-associated protein kinase, *ECM* extracellular matrix, *ERK* extracellular signal–regulated kinase, *FADD* Fas-associated protein with death domain, *HIF* hypoxia-inducible factor, *JAK* Janus kinase, *JNK* c-Jun N-terminal kinase, *MEK* mitogen-activated protein kinase (MAPK)/ERK kinase, *MMP* matrix metalloproteinase, *MSK1* mitogen- and stress-activated kinase 1, *PDK* 3′-phosphoinositide-dependent kinase, *PI3K* phosphatidylinositol 3–kinase, *PIP3* phosphatidylinositol (3,4,5)–trisphosphate, *PKC* protein kinase C, *PLC* phospholipase C, *pRb* phosphorylated retinoblastoma protein, *PTEN* phosphatase and tensin homolog deleted on chromosome 10, *RSK* ribosomal S6 kinase, *SF* scatter factor, *TRADD* tumor necrosis factor (TNF) receptor–associated death domain, *TRAF* TNF receptor–associated factor, *TSP* thrombospondin, *uPA* urokinase-type plasminogen activator, VEGFR2 vascular endothelial growth factor receptor 2. Reprinted with permission from [80]. Copyright 2009 Annual Reviews. All rights reserved

Alterations in Cell-Cycle Regulation

Alterations in pathways that control cell-cycle progression are the most extensively studied in UCa [83]. The cell cycle is primarily controlled by the p53 and Rb pathways, which closely interact with mediators of apoptosis and intracellular signaling.

p53 protein is encoded by the *TP53* tumor-suppressor gene that is located on chromosome 17p13.1 [84]. The protein inhibits cell-cycle progression at the G_1-S transition by activating *p21*$^{WAF1/CIP1}$. While UCa is generally characterized by loss of a single 17p allele, mutation in the remaining allele can lead to *TP53* inactivation and loss of its tumor-suppressor function [85]. Loss of heterozygosity on chromosome 17 occurs in advanced UCa and is associated with an aggressive phenotype. An analysis of high-grade muscle-invasive UCa specimens identified *TP53* mutations in nearly half of the samples, which were mutually exclusive in their relationship with amplification and overexpression of *MDM2*; hence, *TP53* function was noted to be inactivated in 76% of samples [86]. Wild-type p53 has a half-life of <30 min, which prevents its accumulation in the cell nucleus [87]. However, *TP53* mutations result in an altered protein that is resistant to normal ubiquitin-mediated degradation. This causes increased intranuclear p53 accumulation that can be detected by immunohistochemistry [88].

Several retrospective studies have reported that nuclear accumulation of p53 is prognostic in UCa, especially in patients treated with radical cystectomy [89–94]. Altered p53 expression has been shown to increase progressively from normal urothelium to non-muscle-invasive UCa, to muscle-invasive disease and metastatic lymph nodes [95–97]. Despite this evidence, controversy exists on the prognostic role of p53 in bladder tumorigenesis and progression. Indeed, discordance in p53 nuclear accumulation and *TP53* mutations has been documented [98]. A meta-analysis that examined data from 117 studies noted that observational discrepancies may be related to the choice of p53 antibody used in immunohistochemical assays, variability in interpretation and stratification criteria, and other technical and specimen handling inconsistencies [99]. They concluded that current evidence is insufficient to suggest that p53 may be used as a prognostic marker in UCa. A phase III trial designed to evaluate the benefit of stratifying organ-confined invasive UCa patients based on their p53 status for adjuvant cisplatin-containing chemotherapy could not confirm the prognostic value of the protein alteration or any association with chemotherapeutic response [100]. However, this trial was limited by high patient refusal rate, lower than expected event rate, and failure to receive assigned therapy.

The *p21*$^{WAF1/CIP1}$ gene encodes for the p21 cyclin-dependent kinase inhibitor (CDKI). Transcriptionally regulated by p53, the loss of p21 expression is a potential mechanism by which p53 alterations influence tumor progression [72]. Loss of p21 expression is an independent predictor of UCa progression and maintenance of its expression appears to abrogate the deleterious effects of altered p53 [101].

Mdm2 is involved in an autoregulatory feedback loop with p53, thereby controlling its activity. Increased p53 levels upregulate *MDM2* by transactivating its

promoter, and the translated protein mediates proteasomal p53 degradation. The lowered p53 levels then reduce the levels of Mdm2. *MDM2* amplification has been observed in UCa, and its frequency increases with increasing tumor stage and grade [102]. *MDM2* is transcriptionally inhibited by p14. The protein is encoded by *p14^{ARF}*, one of the two splice variants derived from the *CDKN2A* locus located on chromosome 9p21. Because *p14^{ARF}* is induced by the E2F transcription factor, it forms the link between the Rb and p53 pathways [103]. *p14^{ARF}* may be inactivated by homozygous deletion or by methylation of the promoter region [80]. The other splice variant, *p16^{INK4a}*, encodes for p16 that is a CDKI. Homozygous *p16^{INK4a}* deletions in non-muscle-invasive UCa are associated with higher recurrence rates, but deletions that affect both p16 and p14, which deregulate both Rb and p53 pathways, correlate with the worst prognosis [71]. Hemizygous and homozygous deletions of *CDKN2A* have been found in 40–60% and 10–30% of cases, respectively [104].

Encoded on chromosome 13q14, the Rb protein interacts with regulatory proteins involved in the G_1-S transition. Dephosphorylated Rb sequesters the transcription factor E2F. Upon phosphorylation of Rb by cyclin-dependent kinases, E2F is released leading to transcription of genes required for DNA synthesis. Inactivating *RB* mutations resulting in loss of protein expression have been noted in UCa [105]. In conjunction with other cell-cycle regulatory proteins, Rb has also been shown to be prognostic in bladder UCa [92, 93]. Rb phosphorylation is facilitated by cyclin/cyclin-dependent kinase complexes. Negative regulation of cyclin-dependent kinases is achieved by CDKIs such as p21, p16, and p27, which act as tumor suppressors. Low p27 levels have been associated with advanced stage bladder adenocarcinomas [106]. p27 alterations have also been associated with poor disease-free and overall survival in bladder UCa [107]. In the case of patients with pT1 tumors treated with radical cystectomy, p27 alterations in combination with other protein markers improved the predictive value of a nomogram based on standard clinicopathological variables [108]. Combined assessment of p53, p21, Rb, cyclin E1 and p27 has been shown to yield predictive accuracies superior to that of any single molecular marker in UCa patients treated with radical cystectomy, and can improve risk stratification [109, 110].

Apoptotic Pathway Alterations

Apoptosis is a highly regulated process comprising a series of steps that occur throughout normal development and in response to a variety of stimuli resulting in programmed cell death. Apoptosis can be initiated by two pathways. The extrinsic pathway involves activation of cell surface death receptors, and the intrinsic pathway is mediated by mitochondria. Both pathways activate caspases that cleave cellular substrates and result in characteristic apoptotic changes. In vitro tumor-specific caspase-8 expression has been shown to induce apoptosis in urothelial carcinoma cell lines [111]. Decreased caspase-3 expression has also been associated with a higher probability of disease recurrence and cancer-specific mortality [112].

The Bcl-2 family of proteins is involved in the intrinsic apoptotic pathway; it includes antiapoptotic members such as Bcl-2 as well as proapoptotic members such as Bax and Bad. Increased Bcl-2 expression has been associated with poor prognosis in UCa patients treated with radiotherapy or synchronous chemoradiotherapy [113, 114]. Bcl-2 may also serve as a marker in patients with advanced UCa undergoing radiotherapy who may benefit from neoadjuvant chemotherapy [115]. Bcl-2 expression has been associated with decreased tumor-free survival in high-grade T1 disease, and may serve as a good prognostic indicator in non-muscle-invasive UCa in combination with p53 [116, 117]. A prognostic index using Mdm2, p53, and Bcl-2 has also been proposed where aberrations in all three markers corresponded to the worst survival probability in UCa [118]. On the other hand, Bax expression is an independent predictor of a more favorable prognosis in invasive UCa [117, 119, 120]. Bax mediates its proapoptotic role through the activation of Apaf-1 [121]. Decreased Apaf-1 expression has been associated with increased mortality in UCa patients [122].

Alterations in Cell Signaling

Several cell surface receptors modulate signals from external cues and transmit them via transduction pathways to the nuclei of urothelial cells. Alterations in these receptors and/or the transmitted signals can lead to uncontrolled cellular proliferation and tumor formation.

In the FGFR family, activating mutations of *FGFR3* are the most extensively studied alterations in UCa. Nearly 60–70% of low-grade papillary Ta tumors have *FGFR3* mutations [123, 124]. *FGFR3* activation results in downstream signaling through the Ras–mitogen-activated protein kinase (MAPK) pathway. *FGFR3* and *Ras* mutations may be mutually exclusive; nearly 82% of grade 1 tumors and Ta tumors have mutations in either a *Ras* gene or *FGFR3*, suggesting that MAPK pathway activation may be an obligate event in most of these cases [125]. *HRAS* expression has also been associated with noninvasive UCa recurrence at initial presentation [126].

Epidermal growth factor receptor (EGFR) family members include ErbB-1 and ErbB-2 (Her2/neu), which are overexpressed in invasive UCa [127–129]. ErbB-1 overexpression has been associated with higher probability of progression and mortality [130, 131]. Similarly, increased ErbB-2 expression has been associated with aggressive UCa and poor disease-specific survival [132–135]. However, other reports have indicated that ErbB-2 expression is not correlated with prognosis [136, 137]. While the combined expression profile of ErbB-1 and ErbB-2 has been suggested to be a better outcome predictor than each marker alone, this finding has also not been corroborated [138, 139].

Variable expression of sex steroid hormone receptors has been postulated as a potential cause for differential behavior of UCa between genders, although direct evidence to this effect is lacking [13]. Across both genders, decreased estrogen receptor-β expression has been associated with better progression-free survival rates

in patients with noninvasive UCa [140]. Androgen receptor expression has been noted in 75% and 21.4% of patients with non-muscle-invasive and muscle-invasive UCa, respectively [18].

Janus kinase (JAK) constitutes a family of tyrosine kinases that is activated by cytokine and growth receptors and mediates multiple signaling pathways. Increased preoperative plasma levels of interleukin-6, a ligand for the corresponding cytokine receptor, presumably increase JAK signaling and are an independent predictor of UCa recurrence and survival [141]. Following JAK activation, the most well-characterized molecular events include activation of the signal transducer and activator of transcription (STAT) pathway, which control transcription of several important genes. STAT1 can reduce Bcl-2 expression and STAT3 has the opposite effect [142]. *STAT3* expression, in combination with other markers, can predict risk of recurrence and survival in UCa patients [143].

Tumor Angiogenesis

Angiogenesis involves production tumor cell-derived factors that interact with stromal elements to recruit endothelial cells to the site of malignancy and establish a vascular supply, which provides the required nutrients for growth of cancer cells. Angiogenesis is histologically measured by microvessel density, which may be associated with disease-free and overall survival in UCa [144]. Microvessel density quantification may also provide additional prognostic information in UCa patients with p53-altered tumors [145].

VEGFs are angiogenesis-promoting signaling proteins that promote cellular responses by binding to VEGF receptors (VEGFRs). VEGFR2 (KDR/Flk-1) mediates most of the known cellular responses to VEGF. VEGFR2 expression has been associated with increasing UCa stage and muscle invasion [146]. *VEGFR2* expression is also an important determinant for nodal metastasis in UCa patients [147]. VEGF stimulates nitric oxide synthase, which in turn stimulates nitric oxide formation and tumor vascularization. VEGF overexpression in non-muscle-invasive UCa is associated with early recurrence and progression [148]. High serum levels of VEGF are associated with high UCa stage and grade, vascular invasion, CIS, metastases, and poor disease-free survival [149].

VEGF also induces the formation of urokinase-type plasminogen activator (uPA), which degrades the extracellular matrix, thereby facilitating endothelial cell migration and invasion. uPA generates plasmin that stimulates production of basic and acidic fibroblast growth factors (bFGF and aFGF, respectively). Preoperative plasma uPA levels have been associated with disease progression and death from UCa [150]. Urine bFGF levels have been correlated with UCa stage and local disease recurrence [151, 152]. Urinary aFGF levels in invasive UCa patients also show correlation with disease stage [153].

In addition to regulating the cell cycle, p53 plays an important role in angiogenesis by upregulating TSP-1, a potent inhibitor of angiogenesis. Tumors with p53

alterations are associated with decreased TSP-1 expression, and such tumors demonstrate higher microvessel density [154]. Decreased expression of TSP-1 has been associated with lower probabilities of recurrence-free and overall survival in UCa. A combination of angiogenesis-related biomarkers including VEGF, bFGF, and TSP-1 has also been associated with established clinicopathological features of biologically aggressive disease in patients who underwent radical cystectomy for muscle-invasive UCa [155]. On multivariable analyses that adjusted for standard pathological features, bFGF and TSP-1 were identified as independent predictors of disease recurrence and cancer-specific mortality.

Tumor Cell Invasion

The potential of urothelial carcinoma cells to invade the vasculature and lymphatics determines their ability to spread to adjacent structures and metastasize to distant sites. Ubiquitous to all tissues, cadherins are prime mediators of intercellular adhesion. E-cadherin is the prototypic member of the cadherin family, and it plays a critical role in epithelial cell–cell adhesion. Decreased E-cadherin expression has been correlated with higher risk of tumor recurrence and progression, as well as with shorter survival in UCa [122, 156–159].

A tumor's ability to degrade the matrix and invade the basement membrane is facilitated by the actions of several protease families including uPAs and MMPs. Expression levels of transcripts encoding thymidine phosphorylase, an enzyme that promotes MMP production, is 33-fold higher in muscle-invasive UCa than in non-muscle-invasive tumors, and 260-fold higher than in normal bladder [160]. The corresponding protein levels in muscle-invasive tumors are eightfold higher than in non-muscle-invasive tumors and 15-fold higher than in normal bladder tissue [161]. Increased nuclear reactivity of thymidine phosphorylase has been associated with higher risk of non-muscle-invasive UCa recurrence [162, 163]. Increased MMP-2 and MMP-9 expression have been associated with higher UCa stage and grade [164, 165]. Increased expression of MMP-2 can also predict poor relapse-free and disease-specific survival [166]. MMP-9:E-cadherin ratio is prognostic for disease-specific survival in UCa patients [167].

Integrins are transmembrane glycoproteins which, when altered, can promote tumor progression, invasion, and metastasis. They are receptors for proteins such as adhesion molecules and collagen. Intercellular adhesion molecule 1 (ICAM1) is a member of the immunoglobulin superfamily that binds to certain integrin classes. Immunohistochemical studies have revealed that ICAM1 expression is closely associated with an infiltrative histological phenotype [168]. Serum ICAM1 levels have also been correlated with the presence, grade, and size of bladder tumors [169]. *ICAM1* is a member of multimarker models that can predict nodal status in patients with bladder UCa [170]. In normal urothelial cells, the α6β4 integrin is in close relationship with collagen VII, and it restricts cell migration. Loss of polarity of α6β4 expression has been noted in non-muscle-invasive UCa, and muscle-invasive

tumors show either a loss of α6β4 and/or collagen VII expression or a lack of colocalization of the two proteins [171]. Patients with tumors that exhibit weak α6β4 immunoreactivity have better outcomes than those with either no expression or strong overexpression [172]. Overall, molecular markers of invasion are therefore relatively reliable predictors of patient outcome in UCa.

Assessment and Utility of Multimarker Alterations

Alterations in several molecular pathways can, in tandem, influence the pathogenesis of bladder tumors and their ultimate clinical behavior. Analyzing these alterations in combination may therefore provide deeper insight into the pathobiology of the disease, while also generating panels of markers that may be able to better predict patient outcome and treatment response [173]. The advent of technologies that can assess multiple markers in a reliable, efficient, and cost-effective way has led to their adoption for development of prognostic panels [174]. Several studies have quantified finite numbers of molecular targets across several UCa-associated cellular pathways in an attempt to define prognostic signatures [175].

This strategy was used to develop an objective method for predicting recurrence and progression in noninvasive tumors at first presentation, to potentially allow treatment individualization for these patients [126]. A 24-gene panel spanning across relevant cancer pathways was used to profile patients initially presenting with Ta grade 2–3 tumors who belonged to one of three outcome-based groups: those with no recurrence, recurrence or progression within 5 years of follow-up. A multivariable model based on *CCND3* expression showed 97% sensitivity and 63% specificity for identifying patients who recurred. A similar model based on *HRAS*, *VEGFR2,* and *VEGF* identified patients who progressed with 81% sensitivity and 94% specificity.

We have also used this approach to identify molecular alterations associated with progression across all UCa stages, which could potentially supplement disease staging in predicting clinical outcome [143]. The expressions of 69 genes involved in different cancer pathways were assessed on primary UCa specimens to identify a panel of four markers (*JUN*, *MAP 2K6*, *STAT3,* and *ICAM1*) that were associated with disease recurrence and overall survival. Differences in 5-year probabilities for recurrence and survival based on a favorable versus unfavorable profile using this panel were 41% versus 88%, and 61% versus 5%, respectively (both, $P < 0.001$). The prognostic potential of this panel was confirmed on an independent external dataset (disease-specific survival, $P = 0.039$).

As with efforts to characterize bladder cancer subtypes, early studies employing broad transcriptomic profiling resulted in the identification of large prognostic panels. In one effort, 105 bladder tumors were analyzed using oligonucleotide arrays, and support vector machine algorithms were utilized to test the prognostic abilities of the profiled genes [176]. For predicting overall survival, resulting accuracies were 82% and 90% when considering all UCa patients or only those with

muscle-invasive disease, respectively. A 174-probe signature was also attributed to patients with node-positive disease and poor survival.

Researchers from South Korea have employed high-throughput profiling strategies to identify several markers associated with progression of non-muscle-invasive bladder cancer. The group initially identified an eight-gene signature (comprising *S100A8*, *CELSR3*, *PFKFB4*, *HMOX1*, *MTAP*, *MGC17624*, *KIF1A,* and *COCH*) that was associated with disease progression in this patient subgroup [177]. Interestingly, *S100A8* in combination with *IL1B*, *S100A9* and *EGFR* were also identified as important mediators of progression for muscle-invasive bladder cancer in a separate analysis [178]. The group also documented an expression signature of *S100A8*-correlated genes being a strong predictor of progression in patients with non-muscle-invasive disease [179]. A multivariable Cox regression model using a subset of three genes from the original signature (*CELSR3*, *KIF1A* and *COCH*) was also shown to be an independent predictor of non-muscle-invasive bladder tumor progression [180]. Decreased *MGC17624* expression was correlated with disease progression in the original analysis, and its association with *RUNX3* promoter methylation was shown to represent a poor prognostic combination in patients with non-muscle-invasive tumors [181]. Hypermethylation of three other genes (*HOXA9*, *ISL1,* and *ALDH1A3*) was also shown to be an independent predictor of non-muscle-invasive disease recurrence and progression [182].

Decision models based on clinicopathological metrics can provide reasonable prognostic value to influence clinical management [183, 184]. The largest effort to discover and validate a prognostic genomic signature for clinically high-risk bladder cancer to date performed transcriptome-wide profiling of patients with muscle-invasive and/or node-positive UCa, resulting in the identification of a 15-feature genomic classifier that had a prognostic value of 77% on blinded independent validation [185]. The genomic classifier also uniquely reported on the prognostic potential of certain non-protein-coding transcripts, which have now been shown to play important regulatory roles in cancer development [186]. While the prognostic accuracy of a model that comprised clinical variables alone was 78% in the validation set, it improved to 86% when the genomic classifier was added. Performance of the 15-feature genomic classifier was also validated on four independent datasets that confirmed its prognostic potential.

We have also examined the prognostic importance of a panel of nine biomarkers across all UCa stages [122]. In this study, the addition of smoking history to a clinical model improved its prognostic accuracy from 76 to 81%. The prognostic accuracy increased to 85% when information from the biomarker panel was added, which was significantly higher than the clinical model alone ($P < 0.001$) or when combined with clinical and patient smoking variables ($P = 0.018$). Subsequent studies have confirmed that combining smoking information with molecular markers can improve prognostication in UCa patients [187]. These data suggest that multi-marker assessment can yield robust validated prognostic biomarker panels that can identify subsets of UCa patients with varying outcomes. Their performance may be enhanced in combination with clinical and epidemiologic variables, thereby identifying candidates who may need more aggressive management.

Conclusions

Bladder cancer is increasingly being recognized as a disease that cannot be treated exclusively on the basis of pathologic staging; therapeutic strategies need to focus on the molecular alterations in individual tumors. The availability of sophisticated molecular profiling and computational tools has enabled an increased understanding of the events that lead to urothelial tumorigenesis and progression. Future UCa management will employ consensus marker panels that can provide accurate predictions of prognosis and therapeutic response in individual patients.

Targeted therapeutic strategies based on molecular alterations in UCa are now being developed, and those are outlined in subsequent chapters. However, given the multistep process of bladder tumorigenesis, treatment strategies focused on synergism among agents targeting various pathways is the next step towards rational UCa management, with a goal of achieving optimal therapeutic response. Indeed, recent efforts towards characterizing the bladder cancer genome have laid the roadmap towards identifying the potential therapeutic roles for several targeted agents [188]. Stratifying individuals based on risk factors and tumor expression signatures, followed by optimal surgical treatment and interrupting crucial pathway checkpoints through employment of therapeutic agents that target multiple molecular pathways will ultimately lead to effective management of this disease.

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Chapter 2
Grading, Staging, and Morphologic Risk Stratification of Bladder Cancer

Brian D. Robinson and Francesca Khani

Classification of Bladder Cancer

The vast majority (>90%) of bladder cancers are urothelial carcinomas, and the remaining bladder cancers are less common types such as squamous cell carcinomas, adenocarcinomas, small cell carcinomas, and sarcomas. The 2016 World Health Organization (WHO) *Classification of Tumours of the Urinary System and Male Genital Organs* provides an updated and practical framework for how to categorize bladder lesions, both benign and malignant, and a condensed version of that classification schema is provided in Table 2.1 [1]. Unless otherwise specified, the remainder of this chapter will refer to urothelial carcinomas.

Given their rarity, robust treatment and survival data on non-urothelial malignancies are limited. While it is not uncommon for urothelial carcinomas to contain foci of divergent differentiation, most commonly areas of squamous differentiation, these tumors are still considered as primary urothelial malignancies in most studies. Certain histologic variants of urothelial carcinoma are known to portend a worse prognosis, and these are discussed separately; however, the significance of divergent differentiation (e.g., squamous differentiation) is still debated [2–4]. The exception seems to be small cell carcinoma with most studies finding that any component of small cell carcinoma is associated with more aggressive disease [5–9]. For pure squamous cell carcinomas and adenocarcinomas, survival is typically best predicted by TNM stage, but these tumor types tend to present at later stages, thus their general association with worse survival compared to pure urothelial carcinomas [2, 3, 10–14].

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Table 2.1 Classification of bladder cancer

Urothelial carcinoma
Non-invasive
Urothelial carcinoma in situ
Non-invasive high grade papillary urothelial carcinoma
Non-invasive low grade papillary urothelial carcinoma
Invasive
Urothelial carcinoma, NOS
Urothelial carcinoma with divergent differentiation (e.g., squamous, glandular, etc.)
Micropapillary
Plasmacytoid
Nested
Microcystic
Sarcomatoid
Lymphoepithelial-like carcinoma
Clear cell
Lipid-rich
Giant cell
Squamous cell carcinoma
Adenocarcinoma
Small cell carcinoma
Lymphoma/hematopoietic tumor
Mesenchymal malignancy (sarcoma)
Melanoma

Grading of Bladder Cancer

The International Society of Urologic Pathologists (ISUP)/WHO 2004 grading system is currently the most widely used system for grading urothelial neoplasms [1, 15, 16], and it replaced the previous WHO system that was implemented in 1973. In the 2016 WHO *Classification of Tumours of the Urinary System and Male Genital Organs*, only minor changes to the 2004 system were made related to early lesions [1, 17].

Urothelial neoplasia has two general patterns of growth—flat and papillary—and the nomenclature, which incorporates the grade of the lesion, varies depending upon that architectural pattern. For papillary lesions, four basic grades of urothelial neoplasia exist: papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low grade papillary urothelial carcinoma, and high grade papillary urothelial carcinoma. Flat urothelial lesions encompass a spectrum of findings, some of which are neoplastic and some which represent possible early lesions. Urothelial proliferation of uncertain malignant potential (UPUMP) is a newly introduced term that encompasses the prior categories of flat and papillary hyperplasia. Although a subset of UPUMP lesions may represent early neoplasia, many are ultimately reactive processes on follow-up. Urothelial dysplasia is categorized as a flat lesion that shows cytologic and architectural atypia, and although considered to be early neoplasia, a number of these lesions may not progress in the *de novo* setting. Finally, urothelial carcinoma

Table 2.2 Grading of papillary urothelial neoplasia

	Papilloma	Papillary neoplasm of low malignant potential	Low grade papillary carcinoma	High grade papillary carcinoma
Architecture				
Papillae	Delicate	Delicate, occasionally fused	Fused or branching, delicate	Fused or branching, delicate
Organization of cells	Identical to normal	Polarity identical to normal, often thickened, cohesive	Predominantly ordered, minimal crowding and minimal loss of polarity, any thickness, cohesive	Predominantly disordered with frequent loss of polarity, any thickness, often discohesive
Cytology				
Nuclear size	Identical to normal	Uniform, may show minimal enlargement	Enlarged with variation in size, usually <5× size of lymphocyte	Enlarged with variation in size, often >5× size of lymphocyte
Nuclear shape	Identical to normal	Elongated, round to oval, uniform	Round to oval, slight variation in shape and contour	Moderate to marked pleomorphism
Nuclear chromatin	Fine	Fine	Mild variation between cells, occasional hyperchromasia	Hyperchromasia, moderate to marked variation between cells
Nucleoli	Absent	Absent or inconspicuous	Usually inconspicuous	Multiple prominent nucleoli may be present
Mitoses	Absent	Rare, basally located	Occasional, present at any level	Usually frequent, present at any level, may be atypical

in situ (CIS) is the flat equivalent of high grade papillary urothelial carcinoma, demonstrating similar molecular and immunohistochemical features, as well as an increased risk of progression to invasive disease. Tables 2.2 and 2.3 summarize the grading of urothelial neoplasms.

Multiple studies have validated the ISUP/WHO 2004 grading system and shown its ability to predict recurrence, progression, and survival [18–20]. For patients with benign urothelial papillomas, the risk of progression to higher grade disease and death from disease are nearly zero, and the risk of recurrence is only 8–14% [21–24]. PUNLMPs also pose very little threat to survival, but they do have a non-negligible progression rate of 3.7% and a recurrence rate of up to 36% [20, 25–27]. Low grade papillary urothelial carcinomas have yet higher rates of recurrence and progression: 50% and 10%, respectively [20–22]. However, there may be a spectrum of overlap between PUNLMP and low grade papillary urothelial carcinomas, and no study has to date identified unique alterations that distinguish these entities. Finally, high grade papillary urothelial carcinomas frequently recur (60%) and demonstrate progression to higher stage disease (up to 40%) [20, 28–30].

Table 2.3 Grading of flat urothelial lesions

	Urothelial proliferation of uncertain malignant potential	Urothelial dysplasia	Urothelial carcinoma *in situ*
Architecture			
Organization of cells	Polarity intact, but may show thickened urothelium or early tenting and fibrovascular ingrowth	Predominantly ordered, minimal crowding and minimal loss of polarity, any thickness, cohesive	Predominantly disordered with frequent loss of polarity, any thickness, often discohesive
Cytology			
Nuclear size	Uniform, may show minimal enlargement	Enlarged with variation in size, usually <5× size of lymphocyte	Enlarged with variation in size, often >5× size of lymphocyte
Nuclear shape	Elongated, round to oval, uniform but occasional atypia may occur	Round to oval, slight variation in shape and contour	Moderate to marked pleomorphism
Nuclear chromatin	Fine	Mild variation between cells, occasional hyperchromasia	Hyperchromasia, moderate to marked variation between cells
Nucleoli	Absent or inconspicuous	Usually inconspicuous	Multiple prominent nucleoli may be present
Mitoses	Rare, basally located	Occasional, present at any level	Usually frequent, present at any level, may be atypical

For flat urothelial neoplasia, the natural history of low grade lesions is less clear since reproducibility in their diagnosis is poor and surveillance of non-mass-forming lesions is more difficult to monitor cystoscopically and thus document. However, several studies on urothelial dysplasia have shown progression rates ranging from 15 to 19% [31, 32]. Multiple researchers have evaluated the natural history of urothelial carcinoma *in situ* with studies showing recurrence and progression rates of more than 60% and 25%, respectively, in CIS [33–35].

Papillary Lesions

Urothelial Papilloma

Papillomas are benign neoplasms composed of delicate and discrete fibrovascular cores that are lined by normal-appearing urothelium. The fibrovascular cores may be edematous, but they typically are not fibrotic or hyalinized (Fig. 2.1a). The urothelium lining the papillae is of normal thickness (<7 cells) with retained cellular organization and polarization of the nuclei perpendicular to the basement membrane (Fig. 2.1b). The superficial umbrella cell layer is also preserved. Degenerative

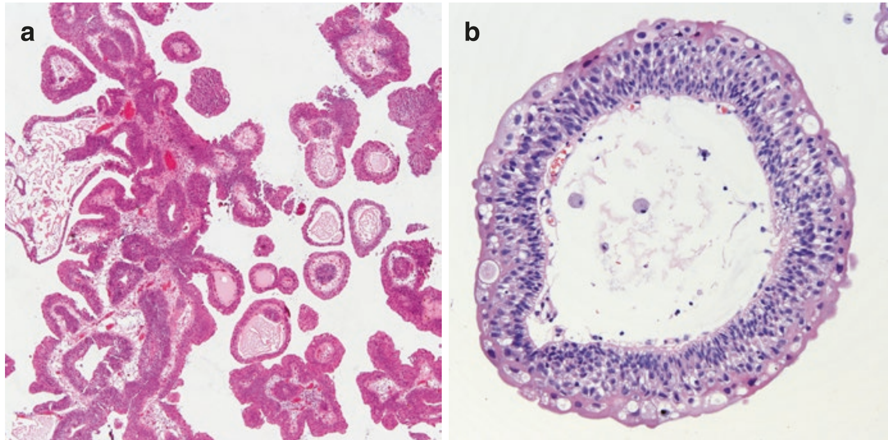

Fig. 2.1 (**a**) All papillary neoplasms in the bladder show an arborizing, frond-like growth pattern. In histologic sections, papillae are often cut in cross-section, and these fibrovascular cores are often edematous in papillomas, as seen here. (**b**) The urothelial lining of the papillae in benign urothelial papillomas is identical to normal urothelium. The cells are cytologically bland and uniform with polarization perpendicular to the basement membrane. The thickness is usually fewer than seven cells, and the umbrella cell layer is preserved and often prominent. [Magnification–40× (**a**), 200× (**b**)]

atypia may be seen in some cases and should not be misinterpreted as carcinoma since papillomas, in contrast to carcinomas, have an excellent prognosis as noted previously. Frequently, vacuolization of the umbrella cell layer is present.

Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP)

The major distinction between PUNLMPs and papillomas is the thickness of the urothelium that lines the fibrovascular cores. Whereas papillomas show a normal (<7 cells) thickness, PUNLMPs show a thickened, hyperplastic urothelium covering the papillae. Retained polarization and minimal atypia is present in the urothelium, such as subtle nuclear enlargement, which is typically diffuse and uniform throughout the lesion. Mitotic figures are rare or absent. PUNLMPs have a slightly higher recurrence rate compared to papillomas but still less than frank carcinomas.

Low Grade Papillary Urothelial Carcinoma

Nuclear atypia and architectural disorganization distinguish papillary urothelial carcinomas from either papilloma or PUNLMP. In low grade papillary urothelial carcinomas (Fig. 2.2a), these features are not overt at low power but recognizable upon closer inspection. The nuclei are enlarged but still less than five times the size of a lymphocyte. Nuclear shape is typically round to oval with some variation of size

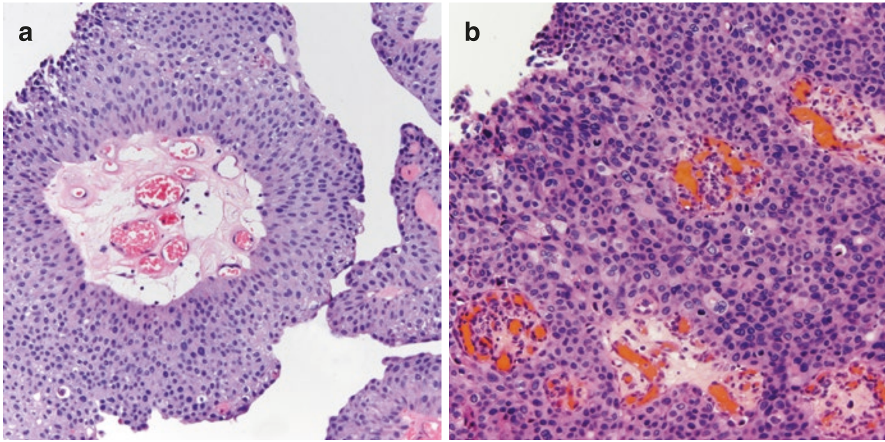

Fig. 2.2 (**a**) Low grade papillary urothelial carcinomas show slight disorganization of cells lining the papillae, but most cells remain aligned perpendicular to the basement membrane. The nuclei may become more rounded, and scattered cells will show nuclear enlargement and hyperchromasia. Mitotic figures may be present. (**b**) In contrast, high grade papillary urothelial carcinomas show complete loss of polarity of tumor cells with nuclear crowding and fusion of papillae. The nuclei are markedly enlarged, hyperchromatic, and pleomorphic with irregular nuclear membranes. Mitotic figures are often numerous, and necrosis may be present. [Magnification–200× (**a**, **b**)]

and shape throughout the tumor (i.e., mild pleomorphism). Nuclear hyperchromasia is also present and variable throughout the tumor with scattered more densely hyperchromatic nuclei present. Nucleoli are generally absent or inconspicuous. Mitotic figures may be present at any level but are typically few in number. In low grade papillary urothelial carcinomas, the urothelium may be of any thickness.

Architectural disorganization in low grade papillary carcinomas is manifest primarily in two ways. First, the papillae are often thicker and more variable in size with some hyalinization and fusion of the cores. Second, the urothelial cells, while still maintaining an overall orderly flow perpendicular to the basement membrane, begin to show some crowding and loss of polarity. When assessing cellular disorganization, it is helpful to look at the spacing of the nuclei. In low grade papillary urothelial carcinoma, it is not uncommon to see clusters of a few nuclei close together and surrounded by large and variable swaths of cytoplasm separating these nuclei from adjacent nuclei.

High Grade Papillary Urothelial Carcinoma

In contrast to lower grade tumors, high grade papillary urothelial carcinomas are marked by extreme nuclear pleomorphism, hyperchromasia, and disorganization (Fig. 2.2b). High grade carcinoma nuclei show marked variability in size and shape with many carcinoma nuclei measuring more than five times the size of a lymphocyte.

Nuclear contours are often irregular and may be angulated. Nucleoli may be prominent and multiple. In addition, mitotic figures are often readily identifiable, present at any level of the urothelium, and may be atypical. The urothelium may be of any thickness in high grade tumors.

High grade carcinoma cells are extremely discohesive, thus resulting in the clear loss of polarity that characterizes these lesions. The cells show no organization with respect to the basement membrane. In some cases, the cells may be so discohesive that the papillae are entirely denuded of urothelium [36, 37]. As with low grade carcinomas, high grade carcinoma papillae are often irregular in size and shape with frequent fusion of cores.

Flat Lesions

Urothelial Carcinoma *In Situ*

Urothelial carcinoma *in situ* (CIS) is the flat counterpart of high grade papillary urothelial carcinoma. That is to say, the same cytologic and architectural features of high grade papillary urothelial carcinoma are present in CIS with the exception of the papillary fibrovascular cores—CIS grows along the normal flattened or undulating bladder mucosa. CIS cells show marked pleomorphism, hyperchromasia, nuclear enlargement (>5× the size of a lymphocyte), and loss of polarity (Fig. 2.3). Similar to high grade papillary carcinomas, the cells of CIS may be so discohesive as to shed from the surface leaving a completely denuded mucosa. In these cases, urine cytology is often helpful in establishing the diagnosis [36].

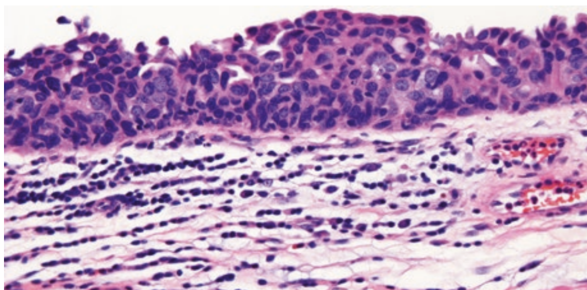

Fig. 2.3 Urothelial carcinoma *in situ* is composed of cells with marked nuclear enlargement (at least 5–6× the size of a stromal cell nucleus or lymphocyte), hyperchromasia, and pleomorphism. The nucleus-to-cytoplasm ratio is increased, and nuclear crowding and overlap of cells is common. The cells also lack any polarization. Mitotic figures and apoptotic cells are commonly seen, and the umbrella cell layer is generally absent. (Magnification–400×)

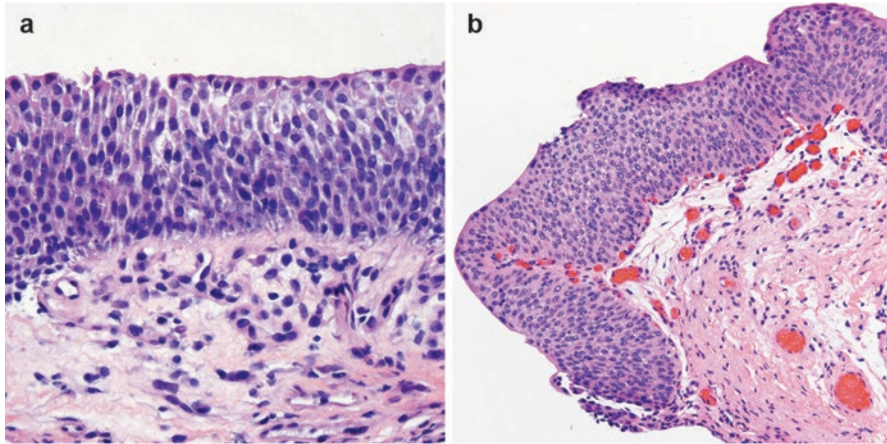

Fig. 2.4 (**a**) Urothelial dysplasia shows atypical features, but these do not reach the level of urothelial carcinoma *in situ* (CIS). The urothelium shows some disorganization; however, overall polarity of the cells perpendicular to the basement membrane is maintained. Some of the cells have enlarged and hyperchromatic nuclei, but this change is not diffuse and generally only moderate (e.g., nuclear size 3–4× the size of a stromal lymphocyte). Mitotic figures and umbrella cell layer may or may not be present. (**b**) Urothelial proliferation of uncertain malignant potential (UPUMP) refers to a cytologically bland to minimally atypical urothelial proliferation that can show increased thickness (generally >10 cell layers) or early tenting of the urothelium. True fibrovascular cores and/or branching are absent. These lesions may be seen *de novo* or encountered in patients with a history of papillary urothelial neoplasia. [Magnification–400× (**a**), 200× (**b**)]

Urothelial Dysplasia

Similar to low grade papillary urothelial carcinomas, and in contrast to CIS, urothelial dysplasia shows only mild nuclear pleomorphism and hyperchromasia. Generally, the nuclei are less than five times the size of a lymphocyte with a round to oval shape and smooth nuclear membranes. Slight disorganization is present but not to the degree of CIS. The overall polarity is maintained perpendicular to the basement membrane, and the cells are cohesive with little denudation (Fig. 2.4a). The umbrella cell layer may be preserved, and mitotic figures may also be present.

Urothelial Proliferation of Uncertain Malignant Potential (UPUMP)

The 2016 WHO Classification System introduced the term “urothelial proliferation of uncertain malignant potential” (UPUMP) for lesions formerly referred to as either flat or papillary hyperplasia [1]. These proliferations are frequently seen in patients with a history of urothelial neoplasia and likely represent an early manifestation of recurrence in these patients [1, 17]. However, their significance in patients without a history of bladder neoplasia remains unclear. Microscopically, the lesions are characterized by thickened but otherwise relatively normal-appearing urothelial

mucosa (Fig. 2.4b). The surface may be flat or undulating and tent-like with what appear to be incipient fibrovascular cores. Well-formed or branching papillary cores are not present. Overall, these lesions are similar to PUNLMPs in cytology and architecture but lack the bona fide papillary fibrovascular cores of PUNLMP.

Invasive Urothelial Carcinoma

The significance of tumor grade in invasive urothelial carcinomas is uncertain as invasive tumors are almost always high grade [1, 17, 38]. Thus, one could argue that the diagnosis of invasion itself is more important than grading invasive carcinoma. When urothelial carcinoma becomes invasive, it usually grows as sheets, trabeculae, or nests of cells with irregular borders and an infiltrative pattern, often with surrounding desmoplastic stroma (Fig. 2.5). In some cases, the tumor may grow as cords or single cells. The cytoplasm of invasive urothelial carcinoma often becomes more abundant and pinker when compared to the overlying non-invasive urothelial carcinoma, which is a phenomenon referred to as paradoxical differentiation. When assessing for superficial invasion and the differential diagnosis includes tangential

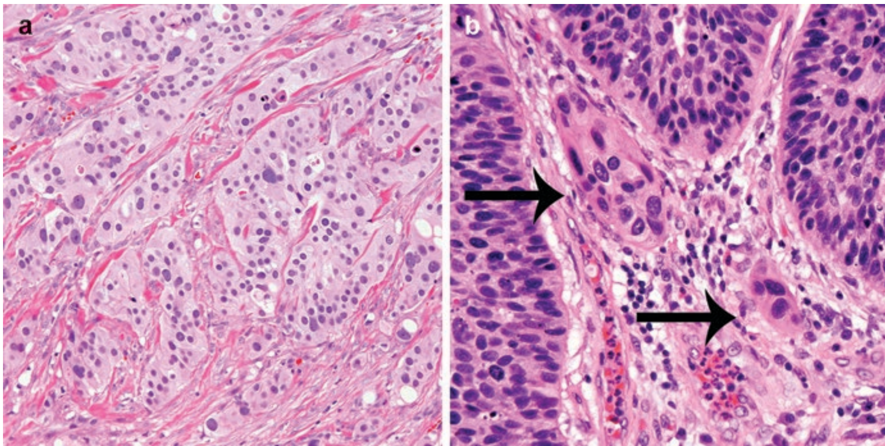

Fig. 2.5 (**a**) Urothelial carcinoma typically invades as nests or cords of high grade pleomorphic cells that elicit a desmoplastic stromal response. An accompanying immune infiltrate may or may not be present. In areas, the tumor may take on a sheet-like growth pattern containing areas of geographic necrosis as the tumor outgrows its blood supply. (**b**) In minimally invasive urothelial carcinomas, the invasive foci may be subtle to detect and/or differentiate from inverted growth. Lack of a basement membrane, irregular borders to the tumor cell nests, and small size of the nests are features of invasive carcinoma. In addition, invasive carcinoma cells often have more abundant bright pink cytoplasm than their overlying non-invasive counterparts. In this image, the *arrows* highlight two nests of invasive carcinoma, and the surrounding nests at the edge of the figure are non-invasive. [Magnification–200× (**a**), 400× (**b**)]

sectioning or inverted growth, the presence of paradoxical differentiation is often helpful, as is retraction artifact [39]. Non-invasive tumors, which are still confined within and attached to the basement membrane, rarely pull away, or retract, from the surrounding stroma during tissue processing.

Staging of Urothelial Carcinoma

The American Joint Committee on Cancer (AJCC) staging system is the most commonly used system for staging bladder cancer, and staging of primary tumors (pT) has not changed in the 8th edition of the AJCC Staging Manual [40]. Unlike almost all other organ systems (except for the penis), non-invasive bladder cancer is separated into two stages, rather than one stage, which mirrors the two architectural growth patterns of urothelial neoplasia: papillary (pTa) and flat (pTis). The remaining stages of invasive bladder cancer (pT1-pT4) remain comparable to other organ systems (Table 2.4).

Table 2.4 Pathologic staging of bladder cancer (AJCC Staging Manual 8th edition)

Primary tumor (T)
Tx—Primary tumor cannot be assessed
T0—No evidence of primary tumor
Ta—Non-invasive papillary urothelial carcinoma
Tis—Urothelial carcinoma *in situ*
T1—Tumor invades lamina propria
T2—Tumor invades muscularis propria
T2a—Tumor invades inner half of muscularis propria
T2b—Tumor invades outer half of muscularis propria
T3—Tumor invades perivesical soft tissue
T3a—Microscopically
T3b—Macroscopically
T4—Tumor invades adjacent structures/organs
T4a—Prostatic stroma, uterus, vagina, rectum, seminal vesicles
T4b—Pelvic wall, abdominal wall
Regional Lymph nodes (N)
Nx—Lymph nodes cannot be assessed
N0—No lymph node metastasis
N1—Single regional lymph node metastasis
N2—Multiple regional lymph node metastases
N3—Metastasis to common iliac lymph nodes
Distant Metastasis (M)
M0—No distant metastasis
M1—Distant metastasis
M1a—Distant metastasis to lymph nodes beyond common iliac lymph nodes
M1b—Distant metastasis to non-lymph-node sites

pTa–Non-invasive Papillary Carcinoma

Tumors that have a papillary configuration or growth pattern but are not invasive are staged as pTa. In these non-invasive tumors, as mentioned previously, the grade of the tumor remains a significant and important prognostic factor. While low grade pTa tumors are likely to recur, progression to high grade or invasive disease is uncommon. In contrast, high grade pTa tumors frequently recur and progress to invasive disease.

pTis–Urothelial Carcinoma *In Situ*

By definition, all pTis tumors are high grade, since urothelial carcinoma *in situ* is a high grade lesion. In pTis tumors, the growth pattern is flat and without invasion of the underlying connective tissue. Recurrence of CIS is common, and progression to invasive urothelial carcinoma is also frequent.

pT1–Lamina Propria Invasion

Invasion into the lamina propria (subepithelial connective tissue) represents the earliest stage in invasive urothelial carcinoma and consequently the best prognosis, with a 5-year survival rate of 88%, which is only slightly lower than pTis tumors [41]. pT1 tumors are those that have broken through the basement membrane and into the lamina propria, which provides tumor cells with access to angiolymphatic channels and thus a potentially worse prognosis when compared to pTa or pTis tumors. Previously, pT1 tumors were substaged based upon the depth of lamina propria; however, most pT1 tumors are diagnosed on transurethral resection (TUR) specimens, which consists of fragmented and unoriented pieces of bladder tissue making such substage assessment difficult and thus led to its abandonment [1]. More recently, several studies have suggested other means of assessing invasiveness of pT1 tumors, and these methodologies are currently under investigation [42, 43].

pT2–Muscularis Propria Invasion

Muscularis propria (detrusor muscle) consists of large bundles of organized smooth muscle that provides the bladder with its contractility. Invasion into these muscle bundles portends a significantly worse prognosis compared to lesser stage bladder cancer and thus is often the indication for radical cystectomy. On TUR specimens, only presence of muscularis propria invasion can be documented; however, at radical or partial cystectomy, where the entire thickness of the bladder wall can be assessed, pT2 tumors are substaged based on whether the cancer invades into the inner half (pT2a) or outer half (pT2b) of the detrusor muscle.

pT3–Perivesical Soft Tissue Invasion

Tumors that spread beyond the muscularis propria and into perivesical fat are considered pT3 tumors. This cannot be assessed on TUR specimens, as mentioned previously, since fat can be seen at all levels of the bladder wall and thus documentation of pT3 disease requires radical or partial cystectomy. Survival rates for patients with pT3 disease are poor with only around 46% of patients alive after 5 years [41]. pT3 tumors are substaged as pT3a if tumor microscopically invades the perivesical soft tissue or pT3b if tumor forms an extravesical mass that is macroscopically visible in the underlying soft tissue.

pT4–Prostatic Stroma, Seminal Vesicle, Uterus, Vagina, Pelvic Wall, or Abdominal Wall Invasion

Bladder cancers that invade through the bladder wall to involve adjacent organs or structures are considered pT4 tumors, which is the highest tumor stage and the worse prognosis (15–40% 5-year survival) [41]. Tumors that invade adjacent organs, i.e. prostate, seminal vesicles, uterus, or vagina, are substaged as pT4a while those that invade the pelvic or abdominal wall are considered pT4b. It should be noted that bladder tumors that grow down the urethra and invade prostatic stroma via this mechanism (without invasion through the bladder wall) should be staged using the system for urethral tumors and thus considered as pT2 [40].

Conclusion

Grading and staging of bladder tumors is based upon decades of research with a multitude of supporting literature, and they continue to be mainstays in treatment decision-making and prognostication of bladder cancer. However, grade and stage remain imperfect tools, and, in the era of personalized medicine, treatment decisions must now go beyond the gross and microscopic appearance of a tumor to also incorporate its molecular features, which is the focus of much of the remainder of this textbook.

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Chapter 3
Genomic Assessment of Muscle-Invasive Bladder Cancer: Insights from the Cancer Genome Atlas (TCGA) Project

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Introduction

There are about 380,000 new cases and 150,000 deaths per year from bladder cancer in the world as a whole [1]. Bladder cancer is unusual among the typical adult malignancies in that both pre-invasive and invasive forms of the disease are common. Non-muscle invasive bladder cancer (NMIBC) is defined as cancer which has not invaded through the smooth muscle layer surrounding the bladder, and makes up the majority (80%) of bladder cancer diagnoses [1]. NMIBC is further subdivided pathologically into low- and high-grade papillary tumors, and carcinoma in situ, a flat intraepithelial

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high-grade tumor [1]. Muscle-invasive bladder cancer is associated with a predictable pattern of pelvic and iliac lymph node metastases and to visceral sites, most commonly lung, liver, and bone. When metastatic to other sites, invasive bladder cancer is rarely curable. Although therapeutic progress in the treatment of metastatic bladder cancer has been very slow for many decades, recently there have been promising reports for both targeted and immune checkpoint therapy [2, 3].

Many previous studies dating back over two decades have identified mutations in a large number of genes in invasive bladder cancer: *TP53* [4], *RB1* [5], *FGFR3* [6], *TSC1* [7], and *PIK3CA* [8, 9]. Bladder cancer has one of the highest somatic mutation rates of any human cancer, surpassed only by melanoma and lung squamous and adeno-carcinoma [10]. Many regions of the genome have also been shown to be common targets of either amplification or loss [1, 10, 11], implicating the presence of either dominantly acting oncogenes or tumor suppressor genes, respectively. More recently, RNA expression profiling with unsupervised clustering has been used by multiple groups to identify distinctive subsets of invasive bladder cancer with differences in clinical behavior, response to therapy, and prognosis [10, 12–16]. In addition, whole exome and whole genome sequencing has been used to characterize the mutation spectrum in bladder cancer in greater detail than was possible previously, with identification of many genes that are commonly mutated in bladder cancer, and appear to contribute to bladder cancer development and/or progression [10, 17–26].

In this chapter, we review the molecular findings made possible by the National Cancer Institute-funded The Cancer Genome Atlas (TCGA) program [10]. Our original report included 130 muscle invasive bladder cancers [10], and was updated previously to include 238 bladder cancers [27]. In this chapter, we present a new analysis of 341 invasive bladder cancers collected through the TCGA program, which were centrally reviewed by a team of genitourinary pathologists to remove those cancers with squamous or other variant histologies.

APOBEC Mutagenesis is the Source of High Mutation Rate in Bladder Cancer

Analysis of 341 invasive bladder cancers confirmed a relatively high rate of non-silent mutation in these cancers, with a mean of 7.9 and median of 5.8 per Mb within coding regions, amounting to 328 protein-coding mutations per cancer. Our earlier study had indicated that APOBEC mutagenesis was the major contributor to mutations occurring in bladder cancer [10]. APOBEC enzymes cause mutations that affect cytosine nucleotides occurring in the nucleotide context T$\underline{\text{C}}$W, and cause conversion to T$\underline{\text{T}}$W or T$\underline{\text{G}}$W (W = A/T) in general [28, 29]. To examine mutational categories and processes in greater detail, we performed Bayesian non-negative matrix factorization (Bayesian-NMF) analysis [30, 31] of the mutations occurring in these 341 bladder cancer specimens (Fig. 3.1). Four patterns of mutagenesis were identified. Two were variations on the well-known

Fig. 3.1 Mutation signature analysis in 340 muscle-invasive TCGA bladder cancers. (**a**) Bayesian-NMF [30] was used to identify five patterns of mutation that occur in bladder cancer genomes. Two of them match the APOBEC pattern, T<u>C</u>W– > T<u>T</u>W or T<u>G</u>W. The uppermost signature, APOBEC1, consists of both C > T and C > G mutations, whereas the next, APOBEC2, consists of only C > T mutations. The third mutation signature is that of CpG > TpG, the fourth is an *ERCC2* mutation signature, and the fifth signature is a POLE signature. The y axis gives the number of mutations of assigned to each signature at 96 tri-nucleotide sequence motifs. (**b**) Graph of the total number of mutations associated with five mutation signatures (*upper panel*) and relative proportion of mutation types (*lower panels*) seen in each TCGA bladder cancer sample

APOBEC mutagenesis signature (denoted as APOBEC1 and APOBEC2 in Fig. 3.1). A third signature (C > T_CpG), characterized by C > T transitions at CpG dinucleotides, is likely due to 5-methylcytosine deamination, and is also well known. A fourth common signature (*ERCC2*) had a relatively even spectrum of base changes and was recently identified to be strongly associated with *ERCC2* mutations [31]. A single specimen had an extremely high level (>4000 per genome) of mutation and had a *POLE* (encodes the catalytic subunit of DNA Polymerase Epsilon) mutation (P286R), known to be associated with extremely high mutation rates. The APOBEC signatures accounted for 66% of SNVs overall, while 8% and 20% SNVs were attributed to C > T_CpG and ERCC2 signatures, respectively.

Recent studies have demonstrated that the APOBEC signature mutations are likely due to *APOBEC3A* activity [32]. These findings indicate that *APOBEC3A* expression and *ERCC2* are the main drivers of mutagenesis and therefore tumor development in bladder cancer. Why these mutation types are so common and cause such a high rate of mutation in bladder cancer is unknown.

We then performed unsupervised clustering of the activity of the four signatures on the 340 cancers to identify four mutation signature clusters, MSig1 to MSig4 (Fig. 3.2). The MSig1 cluster ($n = 26$) consisted of cancers with a high overall non-silent mutation rate (median 23.1 per Mb), whose mutations were nearly all of the APOBEC type. MSig4 ($n = 55$ and median 11.7 non-silent mutations per Mb) consisted of cancers with the highest *ERCC2* signature activity and were highly enriched for *ERCC2* mutations (22 of 33 *ERCC2* mutations, $P < 10^{-11}$, Fig. 3.2). MSig3 ($n = 84$ and median 8.4/Mb) consisted of cancers with intermediate mutation levels nearly all due to APOBEC activity. MSig2 comprised just over half of all cancers ($n = 174$), containing those with a relatively low mutation rate (median 3.4/Mb), the least APOBEC activity, and the highest relative levels of C > T_CpG mutations. Remarkably, patients with cancers in MSig1 had an extraordinarily good 5-year survival of greater than 90%, while MSig4 cluster cancers with the lowest mutation rate had the poorest survival (Fig. 3.3a, $P = 0.0004$). In addition, improved survival was also seen in patients with cancers with high mutation burden (Fig. 3.3d, defined by tertile, $P = 0.0015$), and those whose cancers had high APOBEC mutation load (Fig. 3.3b, defined by tertile, $P = 0.002$). These associations with survival remained significant after correction for the effects of age, histology, node status, and stage. The expected association of survival with tumor stage and nodal status was also seen for these patients (Fig. 3.3c, $P < 0.0001$).

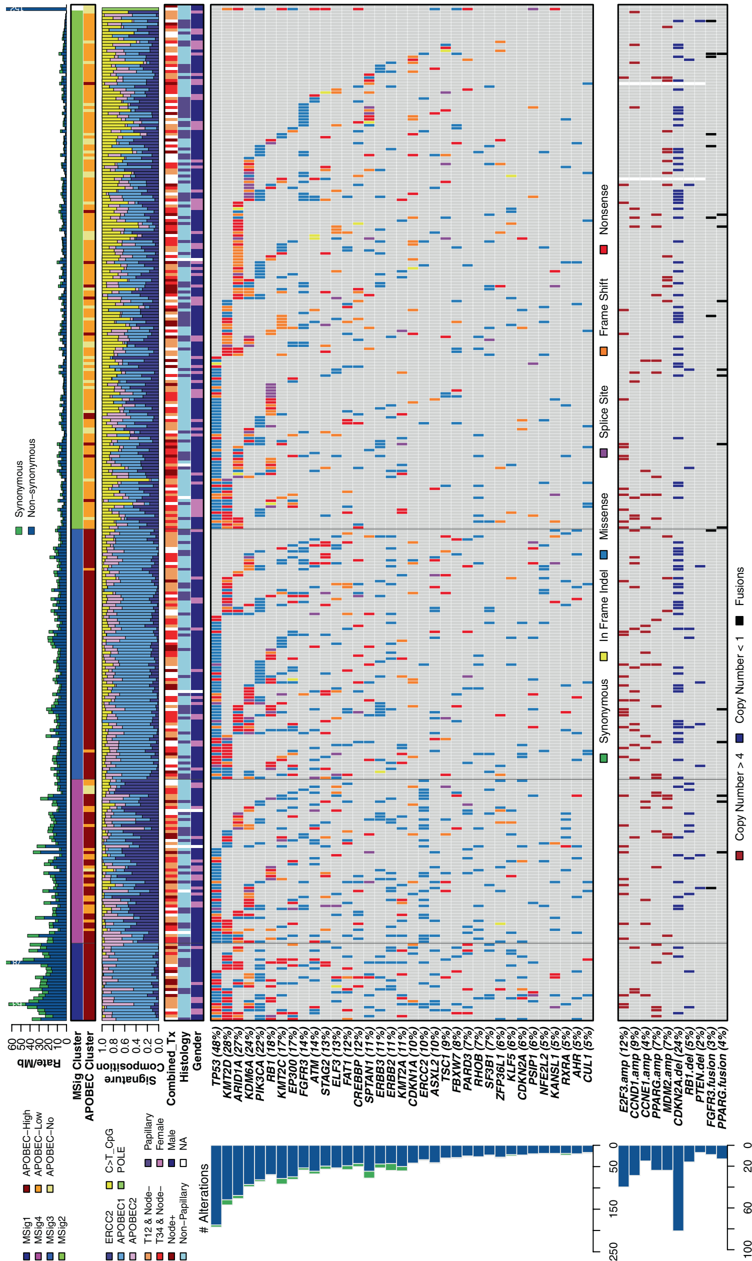


Fig. 3.2 Mutation signature clusters (MSig) and significantly mutated genes (SMGs) in 341 cases of bladder cancer. Mutation data was from the TCGA (341 invasive cases with NOS pathology). Sequentially from top to bottom: mutation rate, mutation signature cluster, APOBEC tertile, fraction of mutations in each mutation signature, combined tumor score, histology, gender, significantly mutated genes with mutation frequency >4%, sorted by mutation frequency, amplifications and deletions for selected genes. Legends for each row of data are shown. *Colors* indicate different mutation types, shown at *bottom*. The total number of mutations and the percent of samples with mutation in each gene is shown at *left*

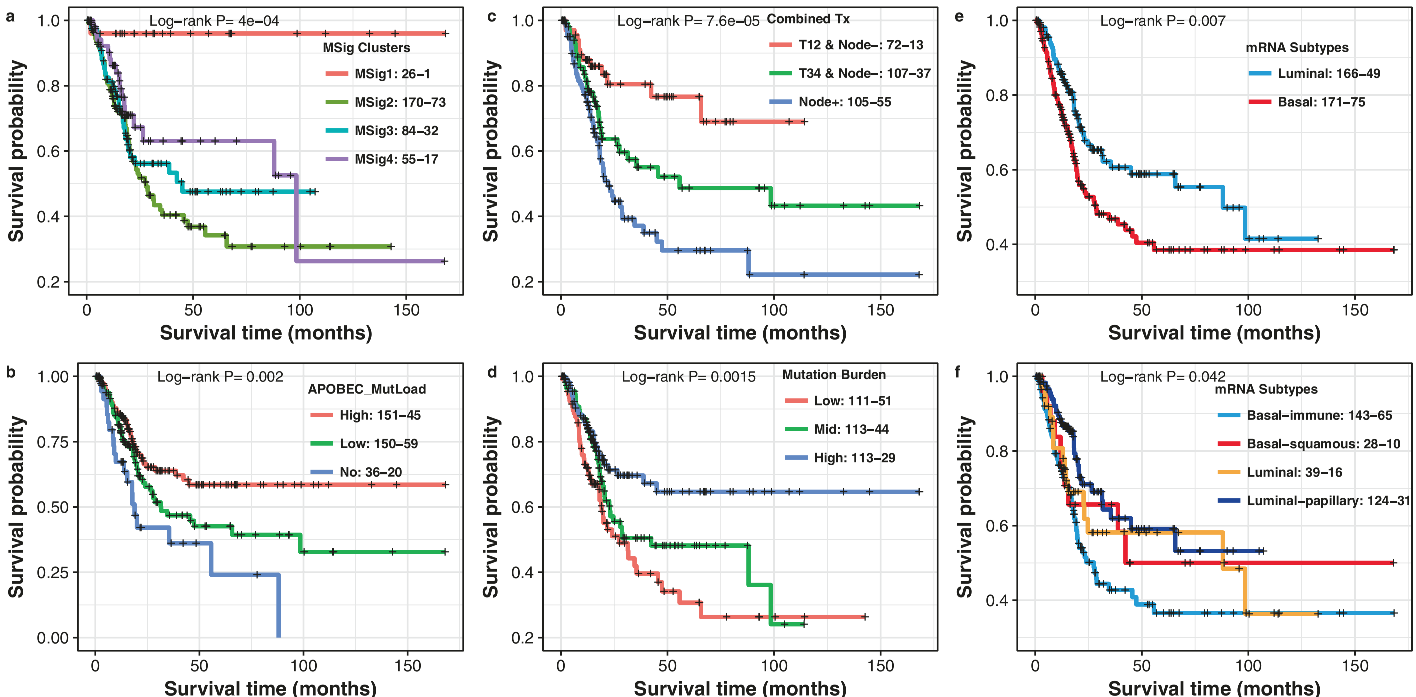


Fig. 3.3 Survival associations for various genetic and expression features. Kaplan-Meier survival curves are shown for 341 subjects categorized in the following ways: (**a**) Mutation signature subgroups (MSig). (**b**) APOBEC mutation load tertiles. (**c**) Tumor-Node (Tx) classification. (**d**) Mutation burden (SNVs) tertiles. (**e**) Basal vs. Luminal expression subtypes. (**f**) Four expression subtypes

Genes Commonly Mutated in Bladder Cancer

We used MutSig 2CV [33] to identify genes that are mutated at a statistically significantly frequency in bladder cancer, so-called significantly mutated genes (SMGs). Fifty-two genes were identified with rates of mutation varying from a high of 48% in *TP53* to a low of 2% in *NRAS* and several other genes (Table 3.1). MutSig takes into account many factors in identifying cancer genes, including gene size and density of mutations [33]. However, it is also important to note that SMGs can change

Table 3.1 Genes mutated or subject to focal copy number change in bladder cancer, and mutual interaction analyses. Genes identified as significantly mutated in bladder cancer (Mutsig 2CV [33]) from analysis of 341 bladder cancers. Sorted in alphabetical order, with number of samples with mutations (percent mutated samples), q value of significance after FDR correction, function, and whether they had been identified previously

Gene	# Mutations (% mutated)	FDR *q*-value	Function	Previously identified in TCGA 130?
ACTB	16 (5)	0.0180696	Motility	False
AHR	18 (5)	0.04969694	Transcription	False
ARID1A	91 (27)	4.68E-11	Chromatin	True
ASXL2	33 (10)	7.27E-05	Chromatin	False
ATM	47 (14)	0.01496431	Cell cycle	False
BAP1	13 (4)	0.01443221	Cell cycle	False
C3orf70	14 (4)	0.00114395	Unknown	False
CDKN1A	35 (10)	4.68E-11	Cell cycle	True
CDKN2A	19 (6)	1.54E-06	Cell cycle	True
CREBBP	40 (12)	2.98E-06	Chromatin	False
CUL1	17 (5)	0.01288072	Proteasome	False
DAZAP1	6 (2)	0.09897366	RNA binding	False
ELF3	43 (13)	3.04E-12	Transcription	True
EP300	57 (17)	4.68E-11	Chromatin	True
ERBB2	38 (11)	0.03868944	RTK	False
ERBB3	39 (11)	0.00689504	RTK	True
ERCC2	33 (10)	1.05E-07	DNA repair	True
FAT1	41 (12)	0.00603335	Cell adhesion	False
FBXW7	26 (8)	3.57E-09	Proteasome	True
FGFR3	47 (14)	3.19E-05	RTK	True
FOXA1	12 (4)	0.00240924	Transcription	True
GNA13	12 (4)	0.00051757	G protein	False
HRAS	16 (5)	1.55E-08	RAS	True
KANSL1	18 (5)	1.65E-05	Unknown	False
KDM6A	83 (24)	4.05E-12	Chromatin	True
KLF5	21 (6)	0.00124881	Transcription	True
KMT2A	37 (11)	0.0021145	Chromatin	False
KMT2C	59 (17)	0.01288072	Chromatin	False

(continued)

Table 3.1 (continued)

Gene	# Mutations (% mutated)	FDR q-value	Function	Previously identified in TCGA 130?
KMT2D	96 (28)	8.61E-12	Chromatin	True
KRAS	15 (4)	2.43E-08	RAS	False
MBD1	11 (3)	0.07222071	Chromatin	False
METTL3	15 (4)	0.09891306	RNA modification	False
NFE2L2	18 (5)	6.67E-06	Transcription	True
NRAS	6 (2)	0.03275192	RAS	False
PARD3	24 (7)	1.46E-05	Cell adhesion	False
PIK3CA	74 (22)	4.92E-12	PI3K-mTOR	True
PSIP1	19 (6)	0.00025831	RTK	True
PTEN	13 (4)	0.0007865	PI3K-mTOR	False
RARS2	7 (2)	0.09891306	Mitochondria	False
RB1	60 (18)	4.73E-12	Cell cycle	True
RHOA	16 (5)	1.46E-05	Motility	True
RHOB	23 (7)	1.01E-12	Motility	True
RXRA	18 (5)	0.0002216	Transcription	True
SF3B1	23 (7)	0.01648582	RNA splicing	False
SPTAN1	39 (11)	8.79E-05	Cell adhesion	False
STAG2	44 (13)	7.31E-11	Chromosome segregation	True
TAF11	7 (2)	0.01752564	Transcription	False
TP53	162 (48)	1.01E-12	Cell cycle	True
TSC1	30 (9)	1.35E-12	PI3K-mTOR	True
ZBTB7B	9 (3)	0.00812552	Transcription	False
ZFP36L1	22 (6)	1.46E-05	Transcription	True
ZNF773	6 (2)	0.09891306	Transcription	False

over time, with some genes no longer statistically significant as a given cancer genome data set expands with more samples. However, with larger numbers of samples typically more SMGs are identified. The 52 SMGs identified participate in a diverse of set of cellular functions, and many have been identified in previous cancer analyses and have known functions whose loss or activation may logically contribute to cancer development. We group the 52 SMGs into multiple functional categories: cell adhesion, cell cycle, chromatin modifying, chromosome segregation, DNA repair, G protein, motility signaling, mitochondria, PI3K-mTOR signaling, proteasome, RAS, RNA binding and modification, receptor tyrosine kinase (RTK), and transcription. Genes involved in cell cycle ($n = 6$), chromatin regulation ($n = 9$), and transcription ($n = 10$) were the most common SMGs. Due to space limitations we cannot discuss all of the SMGs here, but rather focus on several from different functional categories that arguably are of greatest interest.

The p53 pathway was a common target for inactivation in these cancers, seen in 68% tumors overall, with *TP53* mutations in 48%, and *MDM2* amplification in 7% (copy number > 4), and *MDM2* overexpression in 21% (>twofold up c/w median). There was strong mutual exclusivity among these mechanisms of p53 pathway inac-

tivation ($P < 0.00001$ for *TP53* mutation and either *MDM2* amplification or overexpression, Fisher's exact test), indicating that they were independent and sufficient mechanisms of *TP53* functional inactivation. We also observed a significant enrichment of *TP53* mutations in tumors with genome-doubling events ($P < 10^{-7}$, Fisher Exact test), suggesting that loss of TP53 activity may accelerate genome-doubling.

RB pathway inactivation was also common, seen in 53% of these cancers. *RB1* mutations were seen in 18%, were mostly inactivating and associated with reduced mRNA levels ($P < 10^{-15}$, Fisher Exact test), and co-occurred with *TP53* mutations ($P < 10^{-8}$, Fisher's exact test). *CDKN1A* mutations were seen in 10% tumors, and were predominantly inactivating. *CDKN2A* mutations (6% tumors) or homozygous deletions (24% tumors) occurred at reduced frequency in cancers with *RB1* mutation ($P < 10^{-7}$, Fisher Exact test) or *E2F3* amplification ($P = 0.001$), suggesting that mutational inactivation of either gene led to the same functional effect in dysregulation of cell cycle control.

In addition to the *TP53* and *RB* pathways, genomic alterations were common in DNA repair pathways, including mutations in *ATM* (14%) and *ERCC2* (10%), and deletions in *RAD51B* (homozygous deletions in 3%, heterozygous in 13%). All non-silent *ERCC2* mutations were missense, and many of them mapped within or adjacent (+ or −10 amino acids) to the conserved helicase domain, suggesting that these mutations inhibit *ERCC2* function, and may have dominant negative effects [31].

The *FGFR3*, *PIK3CA*, and *RAS* oncogenes all harbored recurrent hotspot mutations in bladder cancer. 26% of all *FGFR3* mutations (seen in 14% tumors) were the well-known S249C or Y375C, and were more frequent in lower stage tumors (22% in T1,2 vs. 8.6% in T3,4: $P = 0.002$). In contrast to most other cancer types, *PIK3CA* mutations (seen in 22% tumors) in bladder cancer were much more common in the helical domain (residues E542 and E545, 53% of mutations) than the kinase domain (residues M1043, H1047, 10%). *PIK3CA* helical-domain mutations were nearly all C > T (86%) or C > G (9%) base substitutions at TCA contexts matching the APOBEC hotspot motif, suggesting they were due to APOBEC activity. Consequently clusters MSig1 and MSig3 with high APOBEC activity (Fig. 3.2) were enriched for *PIK3CA* mutations ($P = 0.0002$ by Fisher's exact test). *ERBB2* mutations (seen in 11%) were more common in an extracellular domain hotspot near S310 ($n = 18$) than in known kinase domain hotspots.

Three low-frequency SMGs, *SF3B1* (seen in 7%), *METTL3* (4%), and *DAZAP1* (2%) are involved in RNA processing, including splicing and modification. SF3B1 had recurrent mutations at codon E902 ($n = 7$), distinct from other known hotspots in uveal melanoma (G742) and breast cancer (K700). The importance of RNA processing gene mutations has been recognized broadly in cancer due to TCGA analyses, and these observations indicate that bladder cancer also has significant frequency of this mutation type.

Nine of the 52 SMGs were either chromatin-modifying or regulatory genes: histone de-methylase (*KDM6A*), histone methyl-transferases (*KMT2A, KMT2C, KMD2D*), histone acetylases (*CREBBP, EP300*), a member of the SWI/SNF chromatin remodeling complex (*ARID1A*), a Polycomb group gene (*ASXL2*), and a transcriptional regulator with a methyl-CpG binding domain (*MBD1*). Mutations in these nine genes were predominantly inactivating (50% frame-shift or nonsense), strongly suggesting that these were not background noise events. *ARID1A, CREBBP,*

KDM6A are also targets of copy number deletion. These observations highlight the importance of chromatin dysregulation in the development of bladder cancer.

Pairwise correlation analysis (Tables 3.2 and 3.3) showed that cancers with mutations in either the *TP53* or the *RB1* pathway had reduced frequency of each of

Table 3.2 Genes mutated or subject to focal copy number change in bladder cancer, and mutual interaction analyses. Genomic events that show pairwise mutual exclusivity in 341 bladder cancers. Specimens. Event1 and event2 are the two genomic events being compared. n1 and n2 are the total number of occurrences of each event, and n.overlap indicates the number of tumors in which both events are seen. Mut denotes mutation; amp focal amplification; del focal deletion

Event1	Event2	n1	n2	n.overlap	*P*-value	FDR *q*-value
RB1.mut	CDKN2A.del	72	91	0	2.34E-09	2.01E-06
TP53.mut	MDM2.amp	198	25	1	9.14E-07	0.00039302
TP53.mut	FGFR3.mut	198	58	13	1.49E-05	0.00427133
TP53.mut	CDKN2A.del	198	91	27	3.76E-05	0.008084
RB1.mut	FGFR3.mut	72	58	2	0.000679	0.116788
E2F3.amp	CDKN2A.del	50	91	3	0.00101	0.14476667
ERCC2.mut	CDKN2A.del	39	91	2	0.00316	0.36597778
ARID1A.mut	FGFR3.mut	101	58	6	0.00346	0.36597778
KMT2A.mut	CDKN2A.del	45	91	3	0.00383	0.36597778
RB1.mut	NFE2L2.mut	72	26	0	0.00569	0.48934
FGFR3.mut	KMT2A.mut	58	45	1	0.00666	0.52069091
RB1.mut	CCND1.amp	72	35	1	0.00845	0.60558333

Table 3.3 Genes mutated or subject to focal copy number change in bladder cancer, and mutual interaction analyses. Genomic events that show pairwise co-occurrence in 341 bladder cancers. Otherwise identical to Table 3.2

Event1	Event2	n1	n2	n.overlap	*P*-value	FDR *q*-value
TP53.mut	RB1.mut	198	72	57	3.15E-09	2.71E-06
TP53.mut	E2F3.amp	198	50	41	1.81E-07	7.78E-05
FGFR3.mut	CDKN2A.del	58	91	27	5.92E-06	0.00169707
FGFR3.mut	STAG2.mut	58	57	20	1.04E-05	0.001978
KDM6A.mut	STAG2.mut	107	57	29	1.15E-05	0.001978
KDM6A.mut	FGFR3.mut	107	58	28	6.33E-05	0.00906686
KMT2D.mut	KMT2C.mut	117	76	36	7.38E-05	0.00906686
PIK3CA.mut	TBC1D12.mut	90	75	28	0.000498	0.053535
PVRL4.amp	YWHAZ.amp	37	36	10	0.000561	0.05360667
KMT2D.mut	FAT1.mut	117	51	25	0.000685	0.05891
SPTAN1.mut	TSC1.mut	48	35	11	0.000854	0.06328167
PPARG.amp	YWHAZ.amp	26	36	8	0.000883	0.06328167

concurrent *CDKN2A* deletions and *FGFR3* mutations. In addition, as described further below, these two sets of mutations were enriched in the basal-squamous ($P = 0.002$, Fisher's exact test) and luminal papillary expression subtypes of bladder cancer ($P < 10^{-6}$, Fisher's exact test), respectively. Thus, this data is highly consistent with classic studies suggesting that bladder cancer can arise either from a pre-existing papillary non-muscle invasive bladder tumor or from a dysplastic carcinoma-in-situ lesion [1].

Somatic Copy Number Alteration (Amplifications, Deletions) in Bladder Cancer

Many previous studies have identified many genomic regions with either amplification or deletion occurring in bladder cancer [1, 10, 11]. In this set of 339 samples, GISTIC analysis [34] of Affymetrix SNP6.0 array data identified many focal somatic copy number alterations (SCNAs), with 35 amplified and 35 deleted regions, that were statistically significant with $q < 0.1$ (Tables 3.4 and 3.5). Many focal amplifications appeared to target genes previously identified in bladder cancer, including *SOX4-E2F3, PVRL4*, YWHAZ, CCND1, PPARG, MDM2*, ZNF703, CCNE1, BCL2L1, MYCL*, ERBB2, EGFR, AHR, FGFR3*, and *KRAS** (genes with * were from regions containing >6 genes, and were the putative targets). Furthermore, several other genomic regions of amplification had not been previously identified, including *GATA3, CTSK*, MMP7*,* and many regions for which the gene (or non-genic) targets are unknown: 16p13.2, 22q12.2, 3q26.33, 17q23.3, 6q21, 4q13.3, 2p25.1, 16q22.1, 19q13.43, 9p24.1, 11p13, 16p11.2, 17q11.2, 17p11.2 (Table 3.4).

The most common recurrent focal deletion (raw copy number < 1), seen in 24% of samples, contained the well-known target *CDKN2A* (9p21.3) and correlated with reduced expression (Table 3.5). Other focal deletions containing <13 genes appeared to target *RB1, PDE4D, CCSER1, CREBBP, LRP1B, FHIT, WWOX, PTEN, RAD51B, FOXQ1*, ERBB4, ARID1A, PTPRD,* and *KDM6A*. As above, most of these had been identified previously but some were novel. In addition, multiple genomic regions were subject to CN loss in this set of bladder cancers, but the specific gene or non-genic target was unknown: 2q37.1, 4q34.2, 8p23.1, 19p13.3, 11p15.5, 18q23, 17p12, 22q13.32, 15q13.1, 6q21, Xq21.33, 6q27, 1q32.1, 11q23.3, 7q36.3, 9q33.2, 12p13.1, 12q24.33, 11q25, and 10p11.21 (Table 3.5).

The majority of genes recognized as targets for amplification or deletion were in the same functional categories as those seen for the SMGs, including cell cycle, chromatin regulation, receptor tyrosine kinase signaling, and transcription.

Table 3.4 Genes and genomic regions identified as being involved in focal copy number amplification, as identified by GISTIC2.0 [45] on 339 bladder cancer samples

Gene	Cytoband	Genomic region	Size (nt)	*q* value
SOX4	6p22.3	chr6:21462625-21757620	294,995	6.99E-83
PVRL4	1q23.3	chr1:161001146-161084006	82,860	7.09E-49
YWHAZ	8q22.3	chr8:101796422-101993580	197,158	5.18E-47
CCND1	11q13.3	chr11:69464719-69481388	16,669	5.80E-44
PPARG	3p25.2	chr3:12408279-12494277	85,998	6.57E-37
MDM2	12q15	chr12:69178021-70150587	972,566	1.92E-25
ZNF703	8p11.23	chr8:37449639-37645465	195,826	5.14E-19
GATA3	10p14	chr10:7924008-8345608	421,600	1.06E-17
CCNE1	19q12	chr19:30290812-30528529	237,717	4.19E-12
BCL2L1	20q11.21	chr20:30109883-30332464	222,581	3.96E-09
MYCL	1p34.2	chr1:40039708-40539227	499,519	9.97E-09
ERBB2	17q12	chr17:37830679-37899687	69,008	5.40E-08
EGFR	7p11.2	chr7:54752424-55428978	676,554	1.16E-06
TERT[a]	5p15.33	chr5:1-2539028	2,539,027	1.20E-06
CTSK[a]	1q21.3	chr1:150496857-150863520	366,663	1.02E-05
LINC00393	13q22.1	chr13:73809522-74092438	282,916	0.000170
	16p13.2	chr16:8524681-9607236	1,082,555	0.000486
AHR	7p21.1	chr7:16925519-17938490	1,012,971	0.000597
	22q12.2	chr22:29404707-31755748	2,351,041	0.000611
	3q26.33	chr3:163842832-198022430	34,179,598	0.00137
FGFR3	4p16.3	chr4:1738268-1817427	79,159	0.00386
	17q23.3	chr17:56860888-81195210	24,334,322	0.00426
	6q21	chr6:107256674-107514789	258,115	0.0150
	4q13.3	chr4:73675242-74840694	1,165,452	0.0195
	2p25.1	chr2:1-10383874	10,383,873	0.0237
MMP7	11q22.2	chr11:101984719-102847937	863,218	0.0438
KRAS	12p12.1	chr12:24880663-26135323	1,254,660	0.0446
	16q22.1	chr16:67091362-76610535	9,519,173	0.0521
	19q13.43	chr19:53202481-58657102	5,454,621	0.0521
	9p24.1	chr9:962233-9621486	8,659,253	0.0684
MYB	6q23.3	chr6:135470481-135807204	336,723	0.0798
	11p13	chr11:33493973-35438140	1,944,167	0.0863
	16p11.2	chr16:28605319-30320319	1,715,000	0.133
	17q11.2	chr17:27064353-27933231	868,878	0.183
	17p11.2	chr17:20571331-25270516	4,699,185	0.269

[a]Gene that is thought to be the target of focal amplification, when there are several in an interval that are also candidates. When no gene is shown, the target of amplification is unknown. The gene symbol, chromosomal cytoband, genomic region, size of the amplified region in nucleotides, and FDR q value are all shown

Table 3.5 Genes and genomic regions identified as being involved in focal copy number deletion. Otherwise identical to Table 3.4

Gene	Cytoband	Genomic region	Size (nt)	*q* value
CDKN2A	9p21.3	chr9:21931610-22003135	71,525	9.14E-204
RB1	13q14.2	chr13:48875329-49563699	688,370	1.65E-33
PDE4D	5q12.1	chr5:58998410-59780946	782,536	4.44E-33
CCSER1	4q22.1	chr4:91271445-93240505	1,969,060	2.09E-24
	2q37.1	chr2:227663186-243199373	15,536,187	2.25E-18
CREBBP	16p13.3	chr16:3675448-4004422	328,974	4.64E-17
LRP1B	2q22.1	chr2:140708948-143637838	2,928,890	1.19E-14
FHIT	3p14.2	chr3:59034763-61547330	2,512,567	1.61E-14
WWOX	16q23.1	chr16:78098006-79031486	933,480	1.54E-13
PTEN	10q23.31	chr10:89617158-90034038	416,880	3.49E-10
	4q34.2	chr4:170674827-191154276	20,479,449	5.61E-09
RAD51B	14q24.1	chr14:68275375-69288431	1,013,056	1.80E-08
	8p23.1	chr8:1-25700626	25,700,625	3.52E-08
	19p13.3	chr19:1-1867911	1,867,910	9.22E-08
FOXQ1	6p25.3	chr6:1-2624052	2,624,051	6.06E-07
	11p15.5	chr11:516521-748945	232,424	5.97E-06
	18q23	chr18:61814593-78077248	16,262,655	2.04E-05
	17p12	chr17:14111288-16121163	2,009,875	3.98E-05
	22q13.32	chr22:47571204-51304566	3,733,362	6.22E-05
ERBB4	2q34	chr2:211542637-214176087	2,633,450	0.000152
ARID1A	1p36.11	chr1:26963410-27155421	192,011	0.000269
	15q13.1	chr15:23689595-32934882	9,245,287	0.000389
PTPRD	9p23	chr9:7887952-12687261	4,799,309	0.00291
	6q21	chr6:67919847-123071476	55,151,629	0.00312
KDM6A	Xp11.3	chrX:44702452-45008304	305,852	0.00541
	Xq21.33	chrX:95661798-99356556	3,694,758	0.0113
	6q27	chr6:152438104-171115067	18,676,963	0.0181
	1q32.1	chr1:188124783-233120332	44,995,549	0.0365
	11q23.3	chr11:102744410-133308731	30,564,321	0.0570
	7q36.3	chr7:151211607-159138663	7,927,056	0.0582
	9q33.2	chr9:125548584-125704087	155,503	0.0673
	12p13.1	chr12:10607226-13714180	3,106,954	0.0920
	12q24.33	chr12:127253242-133851895	6,598,653	0.120
	11q25	chr11:102825636-135006516	32,180,880	0.136
	10p11.21	chr10:17495015-38300099	20,805,084	0.146

Subsets of Bladder Cancer Based upon Expression Profiling

Several recent studies have performed comprehensive gene expression profiling analysis of high grade or muscle invasive bladder cancer and used unsupervised hierarchical clustering to define expression pattern subtypes [10, 12–16]. Although the findings from these studies are not identical, there has been clear similarity and convergence toward a unified model of two distinct types of bladder cancer, basal and luminal. The basal subtype is characterized by expression of primitive keratins *KRT5, KRT14*, and *KRT6A/B/C*, indicative of a basal or stem cell phenotype. The basal subtype was called squamous cell carcinoma-like (SCC-like) by Sjodahl and co-workers [15]. The "luminal" expression subtype, so-called because of its similarity to the breast cancer luminal subtype (also called Urobasal A) [15], is characterized by high expression of *FGFR3*, the uroplakin genes (a marker of umbrella cells), *KRT20*, and transcription factors *PPARG, GATA3, FOXA1*, and *ELF3*. In all studies in which prognostic information has been available, the basal subtype was associated with poorer prognosis, and the luminal subtype was associated with a more favorable prognosis [12–15].

To examine this situation in greater detail, we performed unsupervised expression clustering [35] on the same set of 338 TCGA bladder cancers examined above for mutations and copy number changes (Fig. 3.4). The findings were similar to those published on the 131 samples [10], and identified four different subtypes, splitting the luminal and basal subtypes into two further subtypes each. The four subtypes are labeled basal-immune, basal-squamous, luminal, and luminal papillary. Similar to past studies, the collective basal subtype had worse survival than the collective luminal subtype (Fig. 3.3e), and subdivision into the four subtypes provided further discrimination, with the basal-immune subtype having the worst survival (Fig. 3.3f).

The basal-immune subgroup consists of 145 (43%) samples, and is characterized by high expression of several basal keratin genes (*KRT5, KRT6A, KRT14*), multiple genes characteristic of the p53-like subgroup identified by Choi et al [13]. (*DES, ACTC1, FLNC, PGM5, MFAP4, MYH11, CNN1, ACTG2*), and multiple immune genes (*CCL19, SAA1, NCAM1, LTF, LILRB5, SLAMF6, IL2RA, CD79A, ADAMDEC1, PDCD1LG2*) (Fig. 3.4). Interestingly, this subtype also had high expression of EMT signature genes (*VIM, SNAI2, TWIST1, ZEB1, ZEB2*) and smooth muscle and extracellular matrix genes (*DES, FLNC, MYH11*) consistent with the presence of immune infiltrates and low purity.

The basal-squamous subtype consisted of 28 (8%) samples, and is also characterized by uniformly high expression of several basal keratin genes (*KRT5, KRT6A, KRT14*, even higher than the basal-immune subtype), *DSC3*, *PI3*, and *GSDMC*, indicative of some degree of squamous differentiation. The two basal subtypes lacked expression of classic luminal markers, including urothelium differentiation markers, *KRT20* and UPKs 2/1A/1B/3A.

The two luminal subtypes together had high expression of multiple markers of differentiation toward bladder balloon cells, including *UPK2, UPK1A, SNX31,*

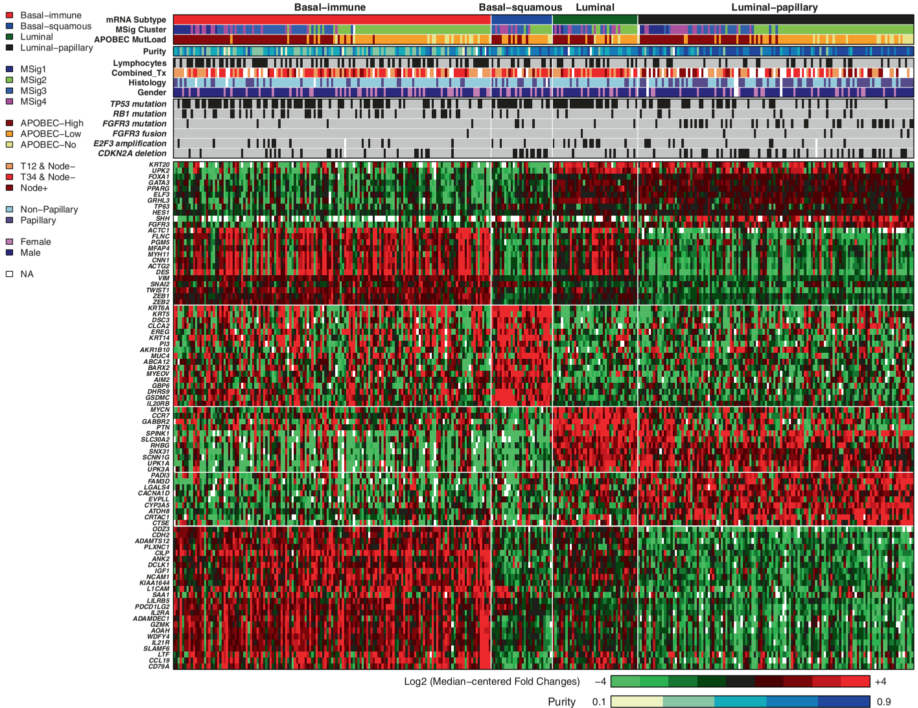


Fig. 3.4 Expression clustering identifies four subtypes of bladder cancer. Unsupervised hierarchical clustering [35] was performed on 338 TCGA bladder cancers using RNA-Seq RSEM expression values for the 3000 most variable genes. Sequential lines going from top to bottom show: mRNA subtype, MSig cluster group, APOBEC mutation load, purity estimate, presence of lymphocytes by pathology review, tumor staging, histology, gender; then presence or absence of mutations in *TP53*, *RB1*, and *FGFR3*; *FGFR3* fusion, *E2F3* amplification, *CDKN2A* deletion. Genes in the top panel of the expression heatmap were selected from the literature [10, 12–16, 27], and include luminal and urothelium-differentiation markers (*KRT20*, *UPK2*, *FOXA1*, *GATA3*, *PPARG*, *ELF3*, *GRHL3*, *FGFR3*), p53-like makers (*ACTC1*, *FLNC*, *PGM5*, *MFAP4*, *MYH11*, *CNN1*, *ACTG2*, *DES*, *VIM*), EMT-markers (SNAI2, TWIST1, ZEB1, ZEB2), squamous-differentiation marker (*TP63*), and sonic-hedgehog markers (*SHH*, *HES1*). The genes in parts 2-5 of the heat map are cluster-specific genes that had a mean expression difference between a single cluster and the other clusters of Log2(RSEM) > 2.5

UPK3A, *KRT20*, all of which were more uniformly up-regulated in the luminal subtype. The luminal subtype also had an intermediate level of EMT marker and stromal gene expression, while the luminal papillary subtype was distinguished from the luminal subtype by enrichment for *FGFR3* mutations and *TACC3-FGFR3* fusions ($P < 10^{-9}$, 43 out of 56), relatively high expression of several genes including sonic hedgehog signaling genes (*SHH*), and genes down-regulated in carcinoma-in-situ (CIS) (*CRTAC1, CTSE, PADI3*).

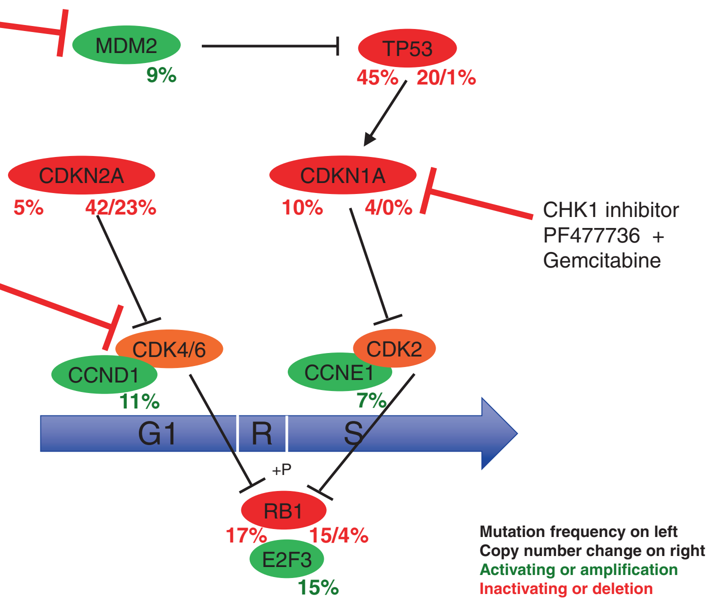
Therapeutic Possibilities in Invasive Bladder Cancer

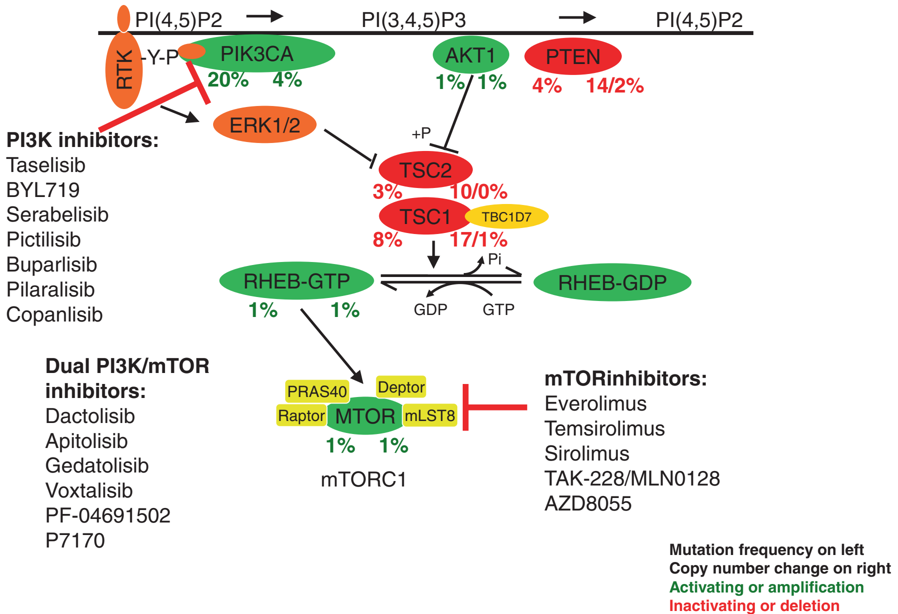
The high mutation rate and frequent copy number alterations lead to many potential therapeutic opportunities for invasive bladder cancer. Fortunately pharma has perceived these same potential targets, and there are many compounds in various phases of pre-clinical and clinical development for the variety of alterations seen.

Specific potential targets include the following, though this is not an exhaustive list. First, mutations and genomic deletions affecting chromatin regulatory genes in bladder cancer are more common than in any other epithelial malignancy [10]. This suggests that therapies targeted at chromatin modifications and epigenetic effects could be useful. Mocetinostat, an oral second-generation HDAC inhibitor, is currently being assessed in a clinical trial for invasive bladder cancers with mutations in either *EP300* or *CREBBP* (NCT02236195) [36]. Further development of agents targeting the effects of these mutations is desired.

Second, mutations and genomic deletion or amplification events that affect the cell cycle are seen in the vast majority of bladder cancer, as noted above. Those include alterations of *TP53* and the cyclin-dependent kinase inhibitors *CDKN1A* and *CDKN2A* (Fig. 3.5a). Both *CDNK2A* loss and amplification of cyclin D1 (gene symbol *CCND1*) can be targeted by agents in development that are *CDK4/6* inhibitors, including palbociclib, abemaciclib, and ribociclib [37]. *MDM2*, amplified in 7% of invasive bladder cancer, is also a therapeutic target of multiple drugs that are in development (Fig. 3.5a). *CDKN1A* mutation, although extremely rare in other cancer types, is seen in about 10% invasive bladder cancer, and occurs with concurrent *TP53* mutation about half the time [38]. Concurrent loss of *CDKN1A* and

Fig. 3.5 Pathways, potential therapeutic targets, and inhibitors for invasive bladder cancer. Genes that drive growth or cancer progression are shown in *green*; genes that are tumor suppressors and act to prevent growth or progression are shown in *red*; kinases without known mutation are shown in *orange*. Beneath each gene symbol, the number on the left indicates the frequency of inactivating (*red*) or activating (*green*) mutation, the number on the *right* indicates the frequency of copy number loss (*red*) or amplification (*green*). Classes of inhibitors and their targets are shown with blunt arrows indicating the components they inhibit. The two numbers shown for copy number loss are for heterozygous (1>CN>0.50, left) and homozygous (CN<0.5, right) loss, respectively (**a**) Cell cycle. (**b**) PI3K-AKT-mTOR pathway. (**c**) RTK-Ras-ERK pathway


a
Cell cycle gene mutations and genomic changes in MIBC
MDM2 inhibitors:
RO5503781 DS-3032b
CGM097
MK-8242
SAR405838
RO6839921
RO5045337
APG-115
RG7388
MDM2
9%
TP53
45% 20/1%
CDKN2A
5% 42/23%
CDKN1A
10% 4/0%
CHK1 inhibitor
PF477736 +
Gemcitabine
CDK4/6
inhibitors:
Palbociclib
abemaciclib
G1T28
Ribociclib
CDK4/6
CCND1
11%
CDK2
CCNE1
7%
G1
R
S
+P
RB1
17% 15/4%
E2F3
15%
Mutation frequency on left
Copy number change on right
Activating or amplification
Inactivating or deletion


b
PI3K-AKT-MTOR pathway in MIBC
PI(4,5)P2
PI(3,4,5)P3
PI(4,5)P2
RTK
-Y-P
PIK3CA
20% 4%
AKT1
1% 1%
PTEN
4% 14/2%
ERK1/2
PI3K inhibitors:
Taselisib
BYL719
Serabelisib
Pictilisib
Buparlisib
Pilaralisib
Copanlisib
+P
TSC2
3% 10/0%
TSC1
TBC1D7
8% 17/1%
Pi
RHEB-GTP
1% 1%
GDP
GTP
RHEB-GDP
Dual PI3K/mTOR
inhibitors:
Dactolisib
Apitolisib
Gedatolisib
Voxtalisib
PF-04691502
P7170
PRAS40
Deptor
Raptor
MTOR
mLST8
1% 1%
mTORC1
mTORinhibitors:
Everolimus
Temsirolimus
Sirolimus
TAK-228/MLN0128
AZD8055
Mutation frequency on left
Copy number change on right
Activating or amplification
Inactivating or deletion

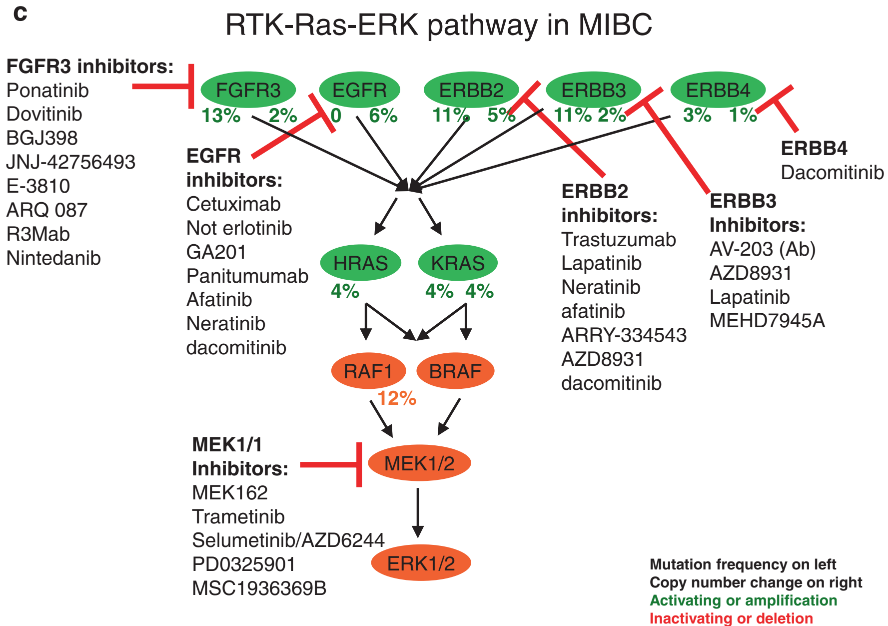


Fig. 3.5 (continued)

TP53 has been shown in cell line and mouse xenograft models to lead to marked sensitivity to combined treatment with gemcitabine and a CHK1 inhibitor, such as PF477736, suggesting potential clinical utility [38].

Third, the PI3K-AKT-mTOR pathway is commonly subject to mutation in invasive bladder cancer (Fig. 3.5b). Mutations are seen in *PIK3CA* (encodes PI3-kinase alpha) in 22%, and multiple agents are in clinical development to target PI3-kinase. Indeed inhibitors have been developed that target a single PI3K isoform, or multiple isoforms, or all isoforms as well as mTOR [39]. Inactivating mutations in *TSC1* are also consistently seen in bladder cancer [7], and they have been shown in at least some cases to lead to dramatic sensitivity to treatment with mTOR inhibitors, such as everolimus [2]. Further studies are underway to define the precise clinical and genetic characteristics of response to mTOR inhibition in bladder cancer. *PTEN* mutations or copy number loss also occur in bladder cancer, and potentially provide therapeutic opportunity.

Fourth, the receptor tyrosine kinase-RAS-ERK signaling pathway is also involved in invasive bladder cancer at significant frequency (Fig. 3.5c). *FGFR3* and all four members of the ERBB family are affected by either activating mutations and/or amplification events (Fig. 3.5c). Drugs that target those genetic abnormalities are at various stages of clinical development. In addition to *FGFR3*-activating mutations (S249C is most common), *TACC3-FGFR3* gene fusions are seen in about 3% of bladder cancer, and both are promising therapeutic targets. Clinical trials of *FGFR3* kinase inhibitors against bladder and other cancers are ongoing [40].

Last, several recent trials have reported promising results in the use of immune checkpoint inhibitor therapy for invasive bladder cancer [3, 41–43]. Two of these reports showed that the anti-PD-L1 monoclonal antibody atezolizumab (MPDL3280A) had significant activity in bladder cancer [3, 43].

The most recent of these reports was a single-arm phase 2 trial, in which patients received atezolizumab 1200 mg given every 3 weeks [3]. The objective response rate was highest, 27%, in those patients with the highest level of PD-L1 expression by their tumor cells and infiltrating immune cells. These results build upon a large and growing body of evidence that immune evasion through cancer-induced immunosuppression, often through activation of immune checkpoints, is an important factor in cancer progression [44]. For example, both cytotoxic T-lymphocyte associated antigen-4 (CTLA4) and programmed death-1 (PD-1) receptor expressed by T cells can be engaged by corresponding receptor molecules on cancer cells (e.g., PD-L1) or other immune cells, to block lymphocyte activity directed at cancer cells [44]. Hence antibodies that block such interaction, directed at either of the interacting molecules, can interfere with cancer checkpoint blockade, leading to native immune cell attack on the cancer, and therefore, to clinical response. The relatively high level of immune gene expression by some bladder cancers, including *CTLA4* and *CD274* (encoding PD-L1) (Fig. 3.4) is consistent with the model that a subset of bladder cancers are characterized by immune suppression, and will be sensitive to immune modulatory therapy. There is continuing investigation of this therapeutic approach, and further evidence in support of this approach for first line therapy of metastatic bladder cancer is possible within a few years. Based upon our analyses of the TCGA expression data, it appears that the Basal- Immune subtype of bladder cancer will be the most promising subtype for immune checkpoint therapy (Fig. 3.4). However, further clinical investigation is required to define the role of these therapies in bladder cancer.

Conclusions

Invasive bladder cancer is characterized by a high overall mutation rate, which appears to be explained mainly by APOBEC-mediated mutagenesis. Cancer genes commonly affected in bladder cancer include both those commonly involved in multiple malignancies and those whose mutation rate is much higher in bladder cancer than in other cancer types (e.g., *CDKN1A, TSC1*). Genes affected include those involved in transcription, chromatin regulation, receptor tyrosine kinase signaling, PI3K-mTOR signaling, RAS, and the cell cycle. Expression profiling studies are consistent in the identification of two main subtypes of bladder cancer, broadly definable as basal and luminal. Basal tumors are less differentiated, more aggressive, and more lethal; luminal tumors are more differentiated and show higher expression levels of uroplakins and *FGFR3*. Expression clustering reveals additional subtypes within the two main groups, and, quite significantly, the subtypes differ in immune gene expression and EMT marker expression.

The future looks bright for therapeutic advances in bladder cancer. Immune checkpoint therapy is particularly promising based on recent clinical trials, and may be most effective in those bladder cancers with high expression of *CD274* (encodes PD-L1), high immune cell infiltrate, and/or a high mutation rate (e.g., MSig cluster 1). However, we also note that bladder cancers with the highest APOBEC mutation signature and mutation rate appear to have a better survival independent of therapy (Fig. 3.3a). There are many other promising targets and drugs that are under current investigation, and will hopefully show positive results in appropriate clinical trials. Promising therapeutic agents directed against the cell cycle, receptor tyrosine kinase pathway, and PI3K-mTOR pathway mutations are in hand. Mutations in chromatin regulatory genes are promising targets for which further pharmaceutical development will be required.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Chapter 4
Molecular Alterations in the Pathogenesis of Bladder Cancer Subtypes and Urothelial Carcinoma Variants

Hikmat Al-Ahmadie and Gopa Iyer

Introduction

Urothelial carcinoma (UCa) is the most common type of bladder cancer but other rare forms of cancer can rarely develop in the bladder including pure squamous cell carcinoma, adenocarcinoma, and small cell carcinoma. UCa is further subdivided into the conventional subtype (usual form) or one of numerous variant histologies. Historically, bladder cancer subtypes and variants of UCa were primarily subdivided based on morphological features. However, recent developments in our understanding of the genomic profiles of these entities have led to a better understanding of the molecular features associated with a subset of these lesions. This chapter will focus specifically on the diagnosis and molecular features associated with the major subtypes of bladder cancer and a subset of UCa variants that are not addressed in other chapters in this text.

Variants of Urothelial Carcinoma

Urothelial Carcinoma with Divergent Differentiation

The most common divergent differentiation in UCa is squamous and glandular differentiation. These two components are typically identified in association with components of the usual urothelial carcinoma.

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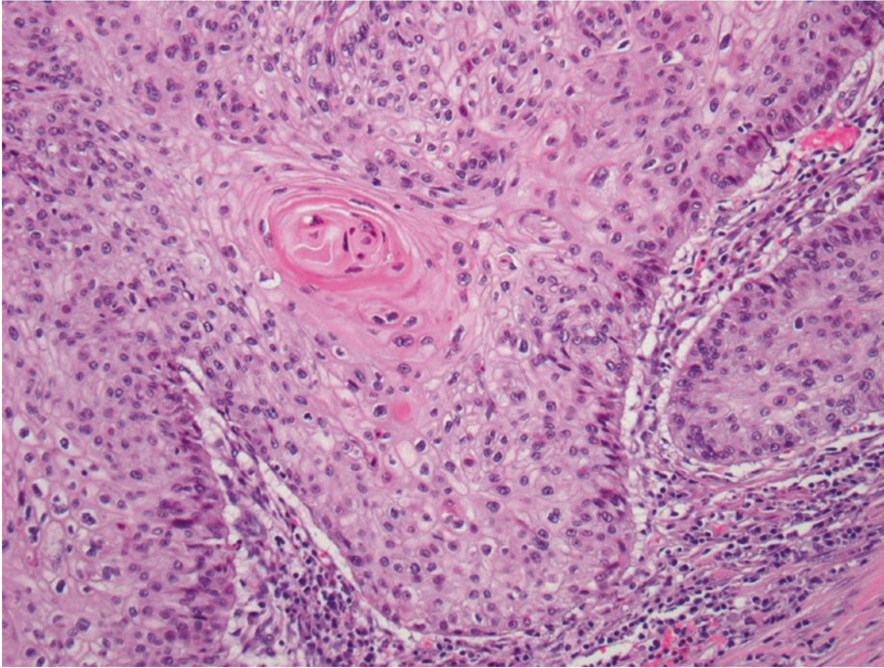

Fig. 4.1 Urothelial carcinoma with squamous differentiation characterized by distinct keratin formation. Squamous differentiation is the most common variant histology in urothelial carcinoma

Squamous differentiation (SqD) is the most common variant histology identified in UCa occurring in up to 40% of cases [1, 2]. SqD in this setting requires the presence of intercellular bridges and/or keratinization (Fig. 4.1). SqD may also be associated with other divergent histologies within an otherwise "usual" UCa, especially in high-grade and high-stage tumors. The term squamous cell carcinoma (SCC) of the bladder should be reserved for tumors that exhibit pure or nearly pure squamous features [2, 3]. SqD is not limited to UCa of the bladder as such morphology can also be seen in UCa of the upper tract [4].

Thorough and careful light microscopic evaluation is the best way to identify squamous lesions but sometimes such distinction may be difficult. There have been a number of markers proposed to aid in this situation but in most times, such markers work best in areas where the light microscopic features are straightforward and may be less helpful in difficult or less straightforward cases.

Both urothelial and squamous areas express many of the same proteins such as p63 and the high molecular weight cytokeratin (HMWK) at high rates [5–9]. Some markers have a tendency to preferentially stain squamous areas such as CK5/6 and CK14 [10, 11]. A recent study reported a novel panel of markers specific for squamous differentiation in a series of primary bladder squamous cell

carcinoma and urothelial carcinoma with squamous differentiation that included MAC387, desmoglein-3, and TRIM29 [12]. These markers preferentially stained squamous cell carcinoma and squamous areas in urothelial carcinoma with squamous differentiation compared to the urothelial areas. Markers that are more likely to stain urothelial than squamous areas include uroplakins, GATA3, S100P, and CK20 [10, 11, 13–20]. It is important to keep in mind, however, that there remains to be some overlap in the expression of these markers in areas of urothelial and squamous features.

The association of human papillomavirus (HPV) and bladder cancer with squamous phenotype has been explored but most evidence points to lack of such association in the vast majority of cases. A few exceptions include patients with neurogenic bladders or those who required repeated catheterization, in which p16 and HPV in situ hybridization was detected in the majority of tumor cells [21, 22]. It is important to note that p16 expression may be seen in conventional urothelial carcinoma with or without squamous differentiation without association with HPV [23]. Expression of this marker is thus insufficient to establish the diagnosis of HPV-associated disease in the absence of HPV genomic integration in the tumor.

A number of studies on the molecular aspects of bladder cancer included cases of UCa with squamous differentiation [24–27]. These studies have revealed robust molecular subtypes of UCa with interesting patterns of gene expression. They all identified a subtype that is enriched with squamous histology. Tumors in this group showed overexpression of high molecular weight keratins (CK5, CK6, and CK14) and epidermal growth factor receptor (EFGR) as well as underexpression of markers of urothelial differentiation such as uroplakins, GATA3, FOXA1, and thrombomodulin. These studies, however, included samples with mixed squamous and urothelial components and as such did not provide a clear evidence to the exact mechanisms involved in the development of the squamous morphology in this setting.

In a separate study comparing the expression profiles of urothelial carcinoma and squamous cell carcinoma of the bladder, Hansel et al. [28] reported the presence of many similarly dysregulated genes and pathways between the two tumor types but there were also many genes that were preferentially dysregulated in the squamous cell carcinoma group particularly those related to squamous-specific morphology regardless of the site of origin (desmosomal complex, squamous epithelium related intermediate filaments, and squamous cornifying proteins).

Glandular differentiation is less common in urothelial carcinoma and the reported incidence is variable in different studies, which is likely related to the subjectivity and familiarity with identifying this variant histology or to selection or referral bias from the reporting institutions. The reported incidence ranges from 8 to 18% [1, 29–31]. The morphology of the glandular component in this setting resembles adenocarcinomas of other organs such as enteric/colonic adenocarcinoma, mucinous or a variety of mixed types (Fig. 4.2). There is limited literature

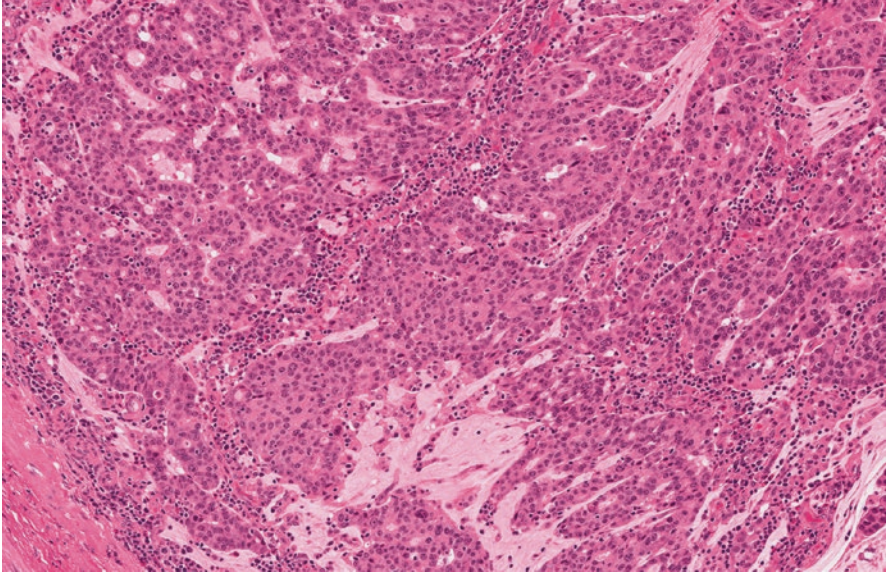

Fig. 4.2 Urothelial carcinoma with glandular differentiation. Tumor with glandular morphology admixed with the urothelial component (*center*)

on the molecular characteristics of glandular differentiation in UCa and they are likely to be overlapping with those of urothelial carcinoma as there is evidence that such tumors similarly harbor hotspot mutation in the *TERT* promoter region [32].

Nested and Microcystic Urothelial Carcinoma

These UCa variants are characterized by the presence of deceptively bland nests of invasive carcinoma that lack significant atypia or stromal reaction (Fig. 4.3). The original description of nested UCa included cases with small nests of invasive tumor but following recent reports, it has been expanded to include the recently described large nested variant and urothelial carcinoma with small tubules [33–36]. Another variant of urothelial carcinoma with bland morphologic features is microcystic UCa which is characterized by the presence of invasive medium-sized cystic structures with bland cytologic features that may show overlapping features with nested UCa [37, 38] (Fig. 4.3). The main challenge in diagnosing these entities is to distinguish them from benign proliferative urothelial conditions including von Brunn nest proliferation, nephrogenic adenoma, cystitis cystica, or inverted papilloma [39, 40]. These variants appear to show similar immunohistochemical features to conventional UCa. As of yet, there is no definitive molecular features associated with these entities to distinguish them from conventional UCa but there seems to be high rate of *TERT* promoter mutations in nested variant of urothelial carcinoma (including

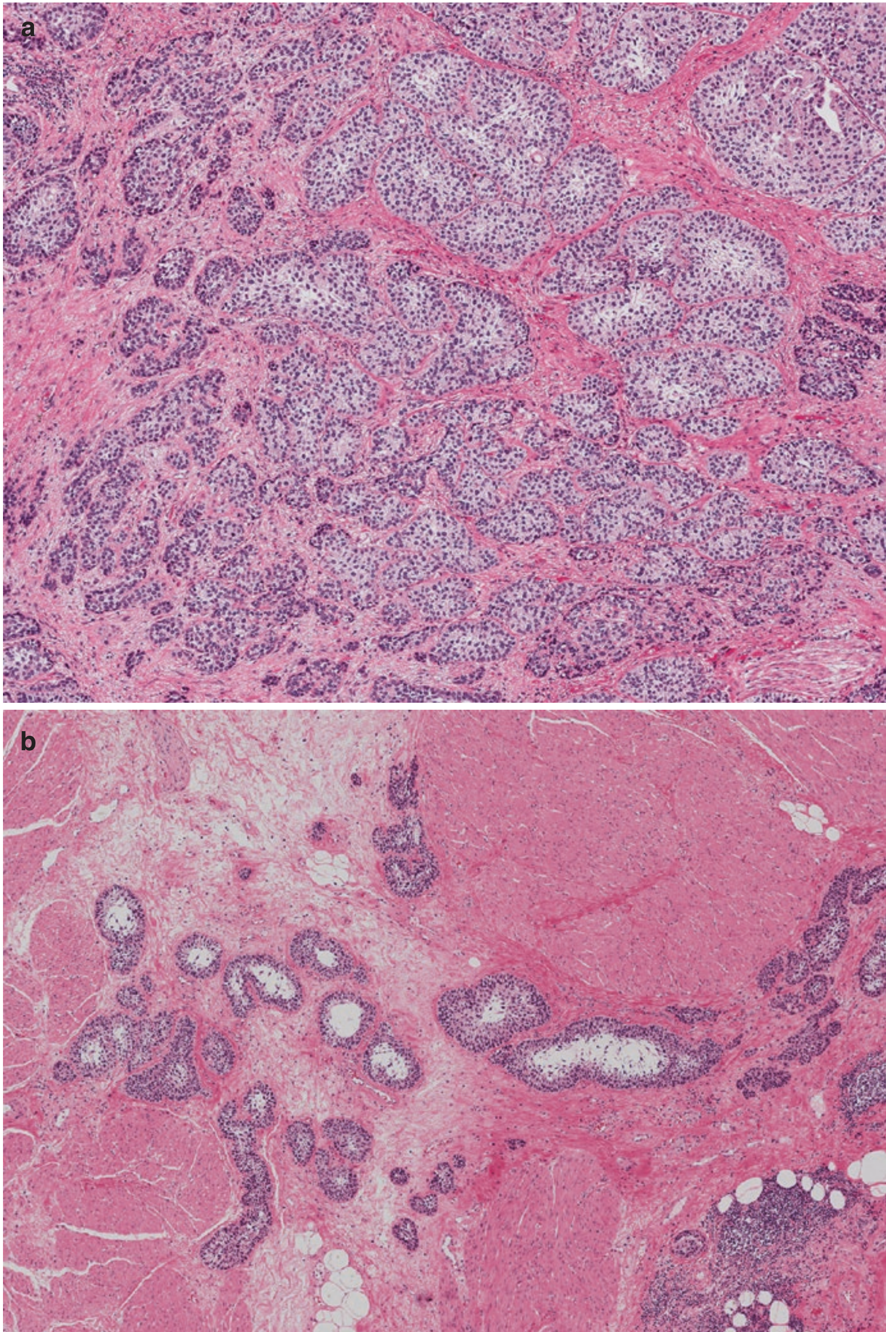

Fig. 4.3 Nested variant of urothelial carcinoma. Variable sized nested of invasive urothelial carcinoma with minimal stromal reaction (**a**). Foci with microcystic morphology and overall bland histology also noted (**b**). This tumor is deeply invasive into the perivesical fat

large nested variant) compared to the absence of such an alteration in benign mimickers [41], which may aid in establishing the diagnosis in challenging cases.

Plasmacytoid Urothelial Carcinoma

Plasmacytoid UCa is a rare but aggressive variant of UCa characterized by the presence of discohesive, individual cells with fair amount of cytoplasm and eccentrically located nuclei that resemble plasma cells [42–44]. In nearly all cases, there is a variable amount of tumor cells with intracytoplasmic vacuoles that give the cells a signet ring cell appearance (Fig. 4.4). This tumor typically follows an aggressive clinical course marked by advanced stage at presentation and association with a high relapse and mortality rate, and frequent peritoneal carcinomatosis despite the apparent initial response to chemotherapy [42–46]. The urothelial nature of this tumor type is supported by immunostains commonly used for urothelial differentiation such as CK7, p63, and uroplakins.

Unlike other variants of urothelial carcinoma (including NOS), it has been recently shown that the presence of truncating mutations or promoter hypermethylation of *CDH1* is the defining feature of plasmacytoid variant of bladder cancer [42]. Using whole exome and targeted sequencing, truncating somatic alterations in the *CDH1* gene were identified in 84% of plasmacytoid carcinomas and were specific to this histologic variant (Fig. 4.4). Furthermore, all but one *CDH1* wild-type plasmacytoid carcinoma exhibited CDH1 promoter hypermethylation and loss of E-cadherin expression. With the exception of *CDH1* mutation, the genomic landscape of plasmacytoid carcinoma was similar to that of UCa, NOS with frequent mutations in chromatin modifying genes, cell cycle regulators, and PI3 kinase pathway alterations [42]. These results suggest that plasmacytoid and UCa-NOS bladder cancers likely evolve from a shared cell of origin. This was further supported by performing exon capture and deep sequencing of two adjacent portions of a bladder tumor which contained distinct regions of plasmacytoid and classic UCa. Both histologic regions shared mutations in *CDKN1A* (A45fs) and *PIK3C2G* (S48R), implying that these were early truncal alterations occurring within a common precursor cell. A *CDH1* Y68fs mutation along with mutations in *PTEN*, *NOTCH2*, *FAT4*, and other genes were, however, unique to the plasmacytoid component [42].

Functional cell lines studies supported a significant role of CDH1 loss in promoting cell discohesion and stromal invasion, which could explain the higher incidence of both local recurrence and cancer-specific mortality as well as the higher rate of peritoneal spread than those with pure urothelial carcinoma. By performing Clustered Regularly Interspersed Palindromic Repeat (CRISPR)/Cas9-mediated knockout of *CDH1* in two *CDH1* wild-type urothelial carcinoma cell lines (RT4 and MGHU4), loss of E-cadherin expression resulted in increased migratory capability of MGHU4 cells. Additionally, both RT4 and MGHU4 CDH1-knockout cells displayed enhanced invasion across a Boyden chamber membrane. These results indicate that somatic loss-of-function mutations in *CDH1*, with consequent

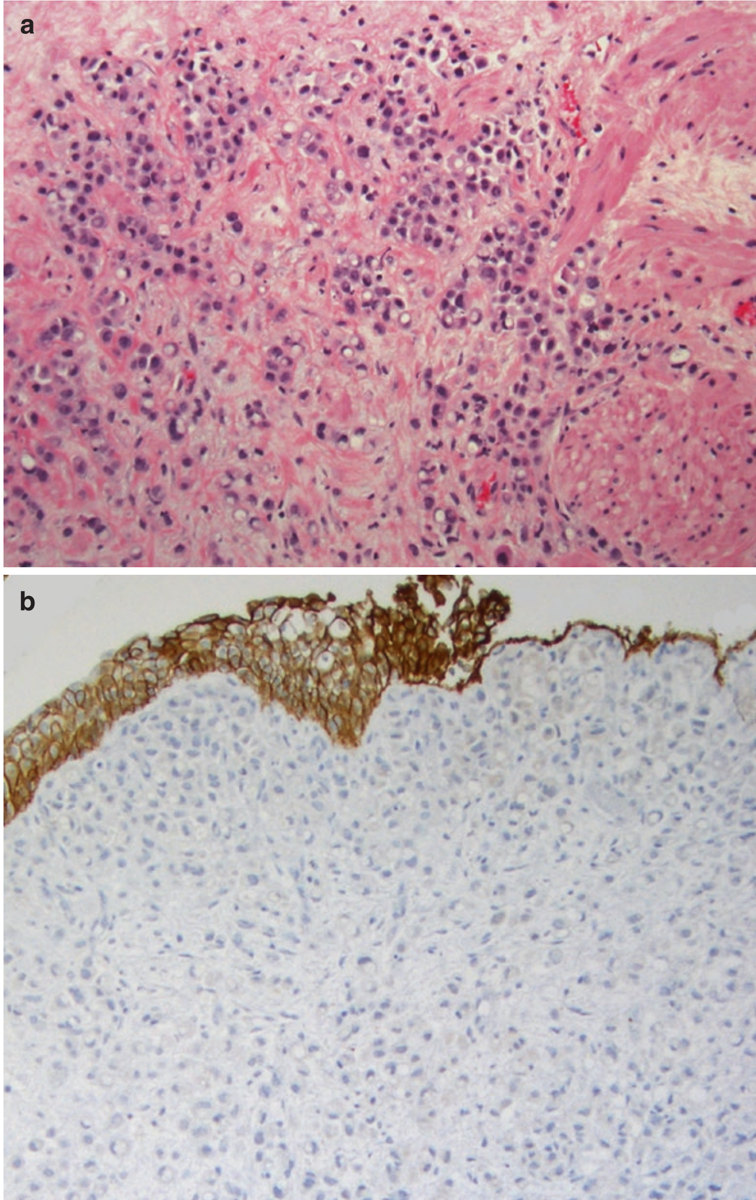

Fig. 4.4 Plasmacytoid urothelial carcinoma with characteristic diffuse and discohesive growth pattern (**a**). Occasional signet ring cells also present. There is complete loss of E-cadherin expression in the invasive tumor (**b**, note E-cadherin retention in the overlying non-neoplastic urothelial mucosa). This tumor harbored a truncating *CDH1*mutation (L729 fs), the gene encoding for E-cadherin. The urothelial carcinoma in-situ component retains membranous E-cadherin expression (**c**, **d**)

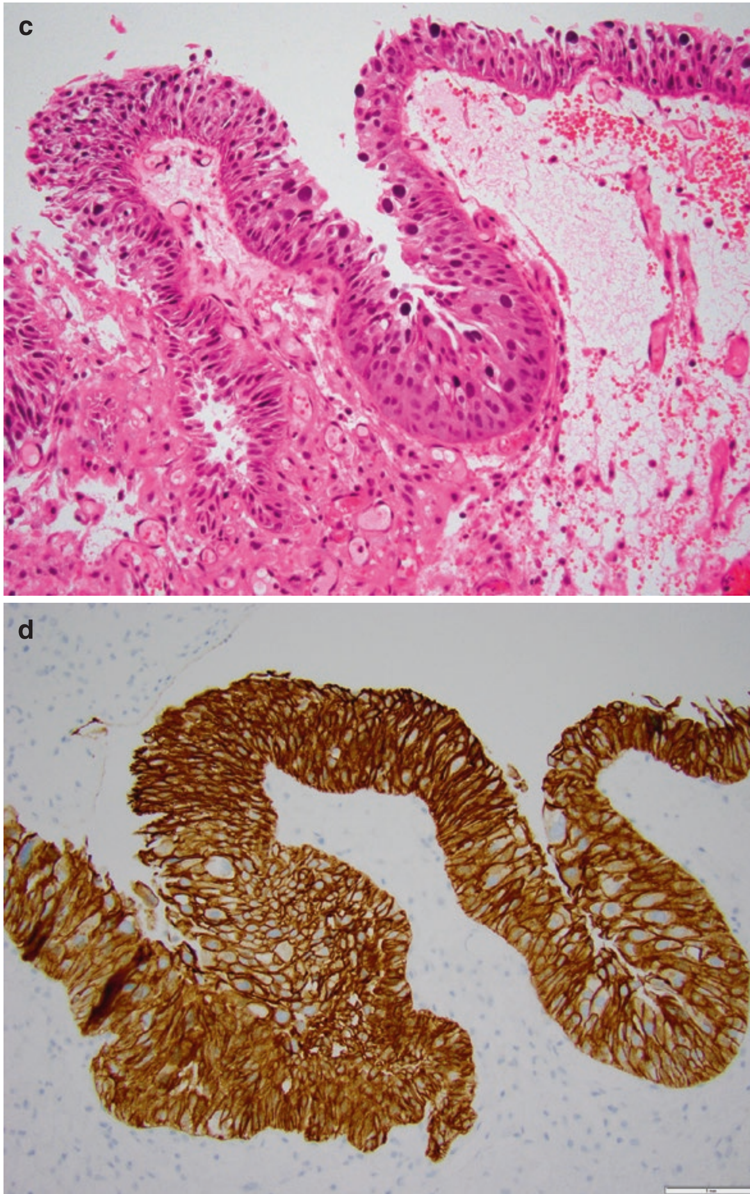

Fig. 4.4 (continued)

E-cadherin loss, lead to enhanced cellular migration and invasive properties in plasmacytoid carcinoma, characterized by marked cell discohesion and single cell infiltration. Notably, E-cadherin staining was absent in the invasive component of plasmacytoid variant tumors but was retained within in situ regions (Fig. 4.4). E-cadherin is a fundamental component of epithelial intercellular adhesions, and E-cadherin loss is implicated in tumor invasion and progression [47, 48], and prior

studies have reported E-cadherin loss by immunohistochemistry is high percentage of plasmacytoid carcinoma [43, 49]. These observations indicate that E-cadherin loss, typically as a result of *CDH1* mutation and less commonly as a result of CDH1 promoter methylation, is the molecular basis for the distinct pattern of local invasion and spread observed in patients with plasmacytoid bladder cancers. Moreover, in contrast to the germline *CDH1* mutations that typify diffuse hereditary gastric cancers and a subset of lobular breast cancer, no germline *CDH1* alterations were identified in the plasmacytoid variant bladder cancer [42].

Micropapillary Urothelial Carcinoma

This is a rare variant of urothelial carcinoma that is now increasingly appreciated but whose diagnosis still lacks high degree of interobserver concordance. This is even more problematic since many clinicians advise early cystectomy for this disease even in the absence of invasion into the muscularis propria [50]. The prevalence of this variant histology is variable ranging from 0.7 to 2.2% in the initial reports to as high as 8% in more recent studies, which may depend on the diagnostic threshold used to identify this variant [51, 52]. The characteristic morphologic appearance of this tumor is that of small tight clusters of tumor cells lacking true fibrovascular cores and present within lacunar spaces (Fig. 4.5) [53]. The basis behind this appearance is the "reverse orientation or polarization" of the basal and luminal aspects of the cells, as shown by electron microscopy as well as MUC1 expression, which is a glycoprotein normally located in the apical aspect of normal glandular epithelium and that is localized predominantly on the stroma-facing surface of the tumor cells in this entity [54, 55]. The end result is the lack of cohesion between tumor and stroma.

Clinically, some studies suggested that conservative treatment for this disease is ineffective and advocated early cystectomy, even in T1 patients while other studies suggest that a more standard bladder sparing approach is reasonable in carefully selected patients in this setting [56, 57]. The application of chemotherapy for the treatment of micropapillary carcinoma showed mixed results with studies showing no benefit from neoadjuvant chemotherapy while others reported efficacy with aggressive systemic chemotherapy [58–60].

At the molecular level, higher rates of *ERBB2* alterations occur in micropapillary carcinoma than in classic UCa, particularly HER2 amplification (Fig. 4.5) [61]. Additionally, a recent study demonstrated that *ERBB2* amplification is associated with worsened cancer-specific survival in patients with micropapillary UC following radical cystectomy [62, 63]. Mutations in known hotspots in *ERBB2* have also been recently reported in micropapillary carcinoma of the bladder [64] but it is not clear whether the frequency of these mutations is higher in this variant histology compared to classic UCa. In another recent study on gene expression profiling of micropapillary bladder cancer, the authors reported the presence of common downregulation of miR-296 and activation of chromatin-remodeling complex RUVBL1 in this disease but did not provide explanation for how these molec-

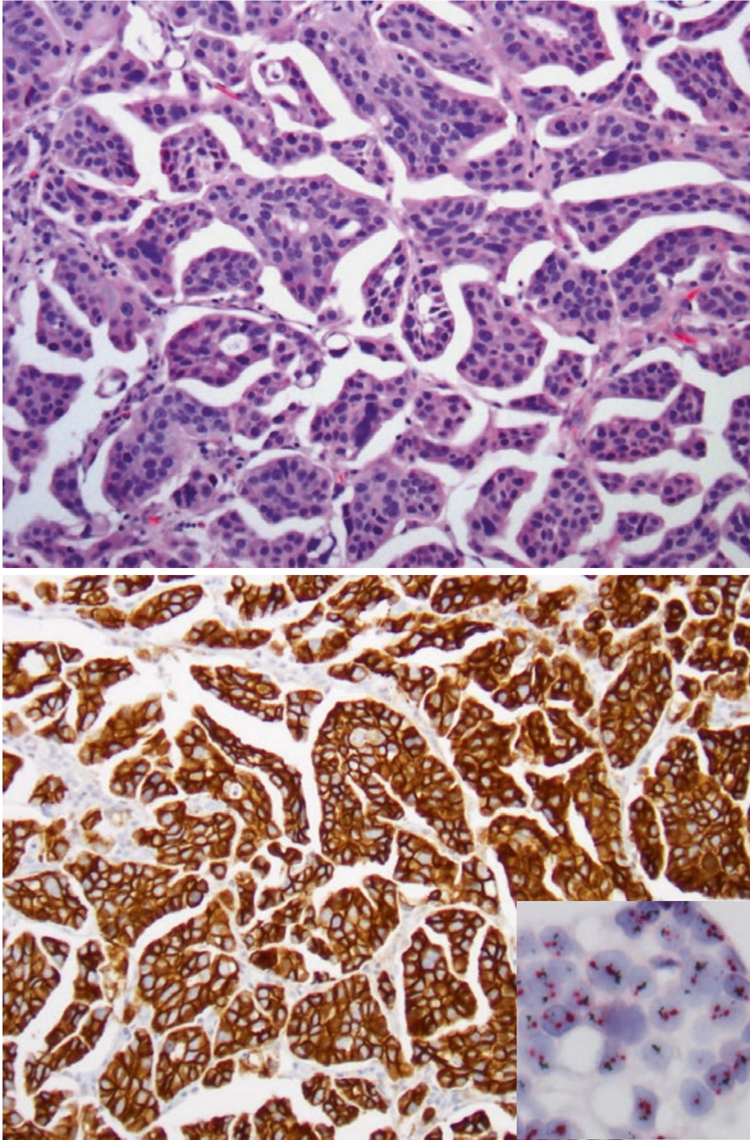

Fig. 4.5 Micropapillary urothelial carcinoma. Clusters of high-grade tumor cells in lacunar spaces (*right*). This variant histology is commonly associated with HER2 overexpression and *ERBB2* amplification as shown by Chromogenic in situ hybridization (CISH) where many copies of *ERBB2* are detected (inset, *brown signal*)

ular events contribute to the development of micropapillary bladder cancer [65]. Interestingly *ERBB2* was one of the genes that were upregulated in the majority of the studies tumors.

Sarcomatoid Urothelial Carcinoma

Sarcomatoid UCa (formerly referred to as "carcinosarcoma") is rare and is usually associated with advanced disease and poor outcomes [66]. This tumor is more common than primary sarcoma of the bladder which is the main differential diagnosis for this entity [1, 67]. Recognizable epithelial morphology is usually present in many of the cases and can represent urothelial, glandular, squamous, and/or small cell/neuroendocrine morphologies. The spectrum of morphologies of the sarcomatous elements is quite variable and may include spindle cell (not otherwise specified), myxoid, pseudoangiosarcomatous, and malignant fibrous histiocytoma-like undifferentiated features. In addition, heterologous elements (osseous, chondroid, etc.) may also be identified in a small subset of cases [1, 68]. It has been shown in earlier studies that the sarcomatous component in this tumor shares common clonal origin with the urothelial component [67]. In a recent study on sarcomatoid urothelial carcinoma, the authors report overexpression of markers of epithelial-to-mesenchymal transition in this tumor including vimentin, FoxC2, SNAIL, and ZEB1, as well as concurrent loss of E-cadherin and elevated N-cadherin expression [68]. Another study reported the presence of frequent *TERT* promoter mutation in sarcomatoid urothelial carcinoma of the upper urinary tract [69]. Similarly, we have encountered cases of sarcomatoid UCa harboring genetic alterations that are similar to those seen in UCa NOS such as mutations in *TERT* promoter, *TP53* and chromatin-remodeling genes (unpublished data, Fig. 4.6).

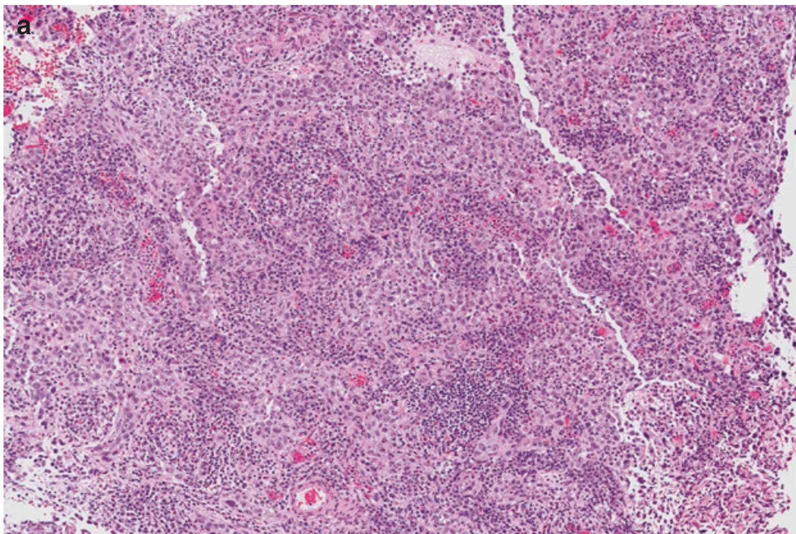

Fig. 4.6 An example of sarcomatoid urothelial carcinoma from a cystoprostatectomy specimen. The tumor consists of high-grade spindle and epithelioid cells with extension to the perivesical fat (**a**). The epithelial component was evident in the transurethral resection specimen (**b**). By targeted next generation sequencing of the sarcomatoid carcinoma the tumor harbored 12 alterations including *TERT* promoter (1295228C > T) mutation and truncating mutations in *TP53* (Q331*) and *ARID1A* (T1921Kfs*16). Alterations in these genes are generally very common in urothelial carcinoma

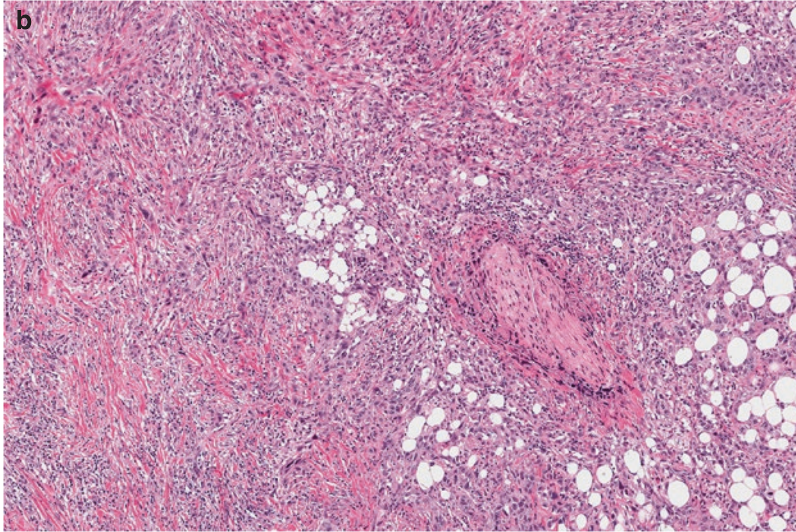

Fig. 4.6 (continued)

Small Cell/Neuroendocrine Carcinoma of the Bladder

This is a rare variant of bladder cancer that is morphologically identical to the small cell carcinoma of the lung, but may be admixed with an epithelial (or rarely sarcomatoid) component of UCa in up to 50% of cases [1]. Epithelial components associated with this tumor are heterogeneous and include urothelial, squamous, glandular morphology or only an in situ component (Fig. 4.7).

The landscape of genomic alterations of small cell bladder cancer is still undefined, yet a few studies have provided intriguing insights into the similarities and differences between small cell and urothelial histology of bladder tumors as well as small cell cancer of the lung. A retrospective sequencing and copy number analysis of 97 carcinomas of the bladder, including ten small cell carcinomas, revealed *RB1* alterations predicted to result in loss of function in every tumor [70], similar to findings in small cell lung cancer [71]. In a second study, 87 matched tumor and germline samples were sequenced from 61 patients with small cell carcinoma of the bladder. Tumors were derived from either transurethral resection (TUR) or cystectomy specimens. Macro-dissection was performed to isolate the neuroendocrine component in those tumors exhibiting mixed histology. Genomic analyses included targeted exon capture, whole exome, and whole transcriptome sequencing. Additionally, two samples were subjected to whole genome sequencing [72, 73]. *TP53* and *RB1* alterations were detected in 90% and 87% of this cohort, respectively, and 80% of tumors displayed co-alterations of both genes, similar to what is observed in small cell lung cancer. Furthermore, loss of expression of RB1 was identified in some tumors without a corresponding loss-of-function mutation,

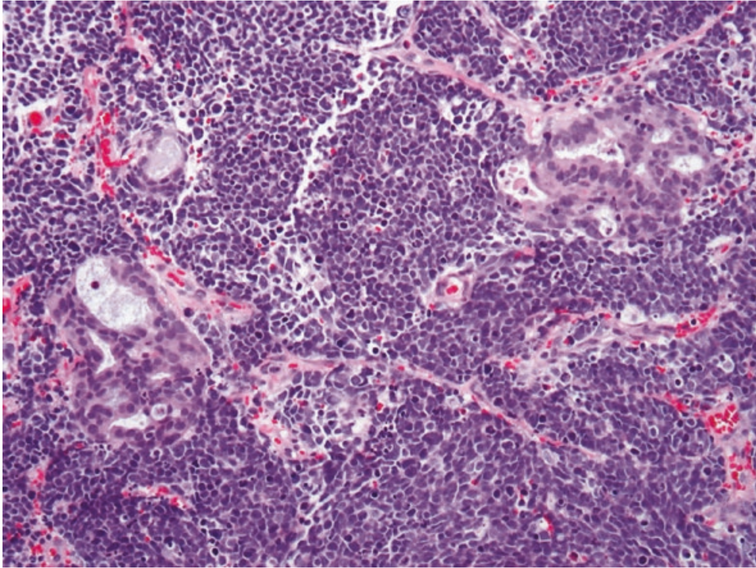

Fig. 4.7 Small cell carcinoma of the urinary bladder. In addition to the small cell/neuroendocrine component, this tumor contains urothelial, glandular, and sarcomatous component (glandular component shown in this figure)

suggesting an alternative mechanism, such as epigenetic silencing, that may contribute to RB1 loss. Moreover, the high frequency of G1/S phase checkpoint disruption indicates that this may be a necessary event in the development of small cell bladder cancer. Interestingly, alterations commonly detected in UCa were also found in the small cell carcinoma cohort, including *TERT* promoter mutations in 95% and truncating alterations within epigenetic modifier genes such as *CREBBP*, *EP300*, *ARID1A*, *KMT2D*, and others, in nearly 75% of samples [72, 73]. A notable exception was *KDM6A* loss-of-function alterations, which were found more frequently in UCa than small cell histology. Activating *FGFR3* mutations, a hallmark of low-grade urothelial tumors and present in approximately 20% of high-grade invasive UCa, were by contrast found in a minority of small cell carcinoma of the bladder. *CDKN2A* deletion and *CCND1* amplifications, found commonly in UCa, were not detected within the small cell carcinoma cohort. *E2F3* amplification was found in both small cell and urothelial bladder tumors, while this event was rare in small cell lung cancer.

A high level of chromosomal instability was observed in bladder small cell carcinoma, including whole genome duplication in 72% of tumors that correlated with the presence of *TP53* missense mutations. The APOBEC mutation signature that was identified within muscle-invasive bladder cancer from the TCGA bladder cancer study [24] was observed in 95% of small cell bladder cancer in this cohort; notably, small cell lung cancers are typically characterized by a mutation signature associated with tobacco exposure distinct from the APOBEC signature.

In a subset of patients, sequencing was performed on the small cell and urothelial components of the same tumor. In two cases, clonal mutations were present that were identified in both the small cell and urothelial histologies, yet *RB1* and *TP53* mutations were sequestered within the small cell histology component, implying that these mutations represent evolutionary branching from a common precursor into two separate histologies. In a second example, clonal mutations within the *TERT* promoter and *PIK3CA* were identified in the small cell and urothelial histologies, while *RB1* and *TP53* alterations were only detected in the small cell component and an ERBB2 L755S mutation only within the urothelial component. These findings clearly support the concept that small cell carcinoma of the bladder is closely related to, and develops form, a precursor UCa. It still remains unclear; however, what exact molecular mechanisms underlie the development of the small cell histology from UCa as much of the reported alterations in small cell carcinoma are similar to what is reported in UCa including the combined *RB1/TP53* which are co-mutated in a subset of UCa that clearly does not display small cell/neuroendocrine differentiation [24, 70, 74].

Due to their rarity, the treatment recommendations for small cell bladder cancers are extrapolated from those for small cell lung cancer, and include systemic cisplatin-based chemotherapy plus radical cystectomy or chemotherapy and radiation therapy. Similar to small cell lung cancer, metastatic spread of small cell bladder cancer occurs early in the disease course and recurrent disease following definitive therapy is typically resistant to additional chemotherapy. Clearly, novel treatments need to be investigated in small cell bladder cancer. Of note, in the cohort described above, 46% of tumors possessed potential therapeutically actionable alterations, including *ERBB2* and *PIK3CA* hotspot activating mutations. The advent of basket trials of small molecular inhibitors, in which patients are enrolled based upon mutation status independent of tumor histology, provides an appealing treatment opportunity for patients with small cell bladder cancer whose tumors harbor such actionable genomic alterations.

Adenocarcinoma of the Bladder

Adenocarcinomas of the bladder as well as urachal adenocarcinomas are rare. While most of these tumors histologically resemble colorectal adenocarcinomas (Fig. 4.8), the genomic alterations that define this rare subset of bladder cancers are not well defined. In one study from a patient with metastatic urachal adenocarcinoma who achieved a long-term (at least 8 months) response to cetuximab (a monoclonal antibody directed against EGFR), targeted exome sequencing of the patient's primary tumor initially identified an amplification of *EGFR* in a *KRAS* wild-type context. Sequencing of nine additional urachal carcinomas revealed MAPK pathway alterations in four tumors and mutations within *APC* in two specimens [75]. An additional cohort of 16 urachal adenocarcinomas was analyzed using a targeted exon capture sequencing approach which revealed *KRAS* hotspot alterations in 5 (29%) and

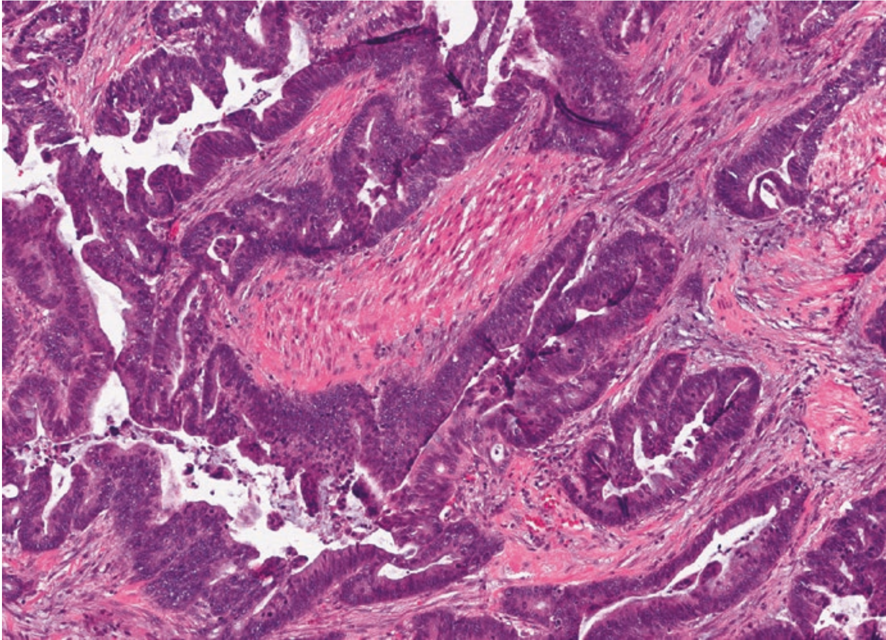

Fig. 4.8 Invasive urachal adenocarcinoma with enteric features, treated with partial cystectomy. This tumor harbored activating *KRAS* (G12D) and a truncating *TP53* (P152fs) mutations in addition to many genetic alterations including *CCND1* amplification and *SMAD4* deletion

ERBB2 activating mutations as well as amplification in 3 (18%) tumors [76]. These results suggest that MAPK pathway activation is a common phenomenon in urachal adenocarcinomas; moreover, this genomic profile of *EGFR* amplification, *APC* mutations, and *KRAS* activating mutations resembles that of colorectal adenocarcinoma. *SMAD4*, a tumor suppressor gene involved in TGF beta signaling, is commonly inactivated in pancreatic and colorectal adenocarcinomas, resulting in activation of the TGF beta pathway. Alterations in *SMAD4*, including two truncating mutations, were observed in 18% of urachal adenocarcinomas in this cohort. Additionally, *GNAS* hotspot alterations and amplification were identified in 18% of tumors. *GNAS* encodes the alpha subunit of the trimeric G protein coupled receptor complex that can activate the MAPK pathway. In a second patient with metastatic urachal adenocarcinoma that had progressed on chemotherapy, activating mutations were detected in *KRAS* (Q61L) and *GNAS* (R201C). Based upon this genomic profile, the patient was initiated on the MEK inhibitor trametinib for compassionate use and achieved over 29 months of stable disease. This response, in combination with that seen with cetuximab therapy, suggests that adenocarcinomas of the bladder and urachus represent a unique opportunity for MAPK pathway inhibition to derive meaningful clinical benefit. These observations also suggest that the genomic landscape of adenocarcinomas of the urinary tract may represent colorectal adenocarcinomas more closely than UCa.

In a separate cohort of nine primary bladder adenocarcinomas, a similarly high rate of *KRAS* alterations (43%) was observed. One specimen harbored *ERBB2* amplification. Interestingly, mutations in *ARID1A* and *SMARCA4*, epigenetic modifiers that are commonly altered in UCa, were also seen. In both urachal and primary bladder adenocarcinomas, *TP53* was the most commonly mutated gene [76].

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Chapter 5
Treatment Paradigms in Bladder Cancer: Clinical Implications of Histological and Molecular Analysis

Mehrad Adibi and Colin P. Dinney

Abbreviations

BCG	Bacillus Calmette Guerin
CIS	Carcinoma in situ
CTLA 4	Cytotoxic T-lymphocyte associated protein 4
EORTC	European Organization for Research and Treatment of Cancer
FDG	^{18}F–fluorodeoxyglucose
FGFR	Fibroblast growth factor receptor
IFN	Interferon alpha
MIBC	Muscle-invasive bladder cancer
MMR	Mismatch repair
MVAC	Methotrexate, vinblastine, adriamycin, and cisplatin
NAC	Neoadjuvant chemotherapy
NBI	Narrow band imaging
NMIBC	Non-muscle invasive bladder cancer
PD-1	Programmed cell death receptor 1
PD-L1	Programmed cell death receptor ligand 1
SWOG	Southwestern Oncology Group
TCGA	The cancer genome atlas
TURBT	Transurethral resection of bladder tumor

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Introduction

Bladder cancer is the second most common genitourinary cancer and the 5th overall most common cancer in the United States, afflicting close to 77,000 patients in 2016, and leading to over 16,000 deaths [1].The vast majority of bladder cancers are urothelial in origin, and roughly 75% present as non-muscle invasive (Ta, T1, CIS), whereas 25% are muscle-invasive or metastatic at initial presentation [2]. Among non-muscle invasive bladder cancers (NMIBC), 50% are low-grade in nature with minimal chance for progression, although roughly 10–15% eventually progress to high grade disease, potentially leading to muscle-invasive bladder cancer (MIBC) [3]. Due to its often long natural history and expensive diagnostic and therapeutic pathways, it is also one of the most costly cancers to diagnose, stage, and treat. It is estimated that the total costs of bladder cancer-related care will amount to >3% of all cancer-related medical payments by the end of the decade [4]. As it stands, the current treatment paradigms for bladder cancer from the time of initial presentation to diagnose and treatment involves multiple complex and often invasive steps that are not only time-consuming and costly, but are often not performed in a standardized and uniform manner among urologist and other healthcare professional responsible for treating this patient population, further adding to the potential downstream effects of increased morbidity and higher economic burden from both a patient and overall healthcare economic perspective. There is evidence to show marked under-utilization and non-adherence to evidence based guidelines, mostly due to provider-level non-compliance when treating this patient population [5]. As a result, a significant paradigm shift is necessary to not only unify and standardize diagnosis and treatment pathways for bladder cancer, but to incorporate precision molecular medicine to decrease unnecessary or ineffective treatments resulting in reductions in patient morbidity and overall healthcare costs.

The current standard of care pathways and algorithms commonly implemented in the treatment of bladder cancer in all of its stages has suffered from a long-standing stagnation with regard to implementing precision molecular medicine in order to individualize patient-specific treatments. Although there are tremendous efforts being made in the characterization of the molecular pathogenesis on a genomic and epigenetic level, when compared to other malignancies, the field of bladder cancer therapeutics has so far lagged behind in implementing translational oncology to many of the gold-standard treatments currently in use. As an example, colorectal cancer, which has arguably undergone one of the most rapid and effective changes with regard to translating basic science efforts to the bedside among all fields in oncology, continues to modify approaches to the screening, diagnosis, and treatment of the disease. As one of the first malignancies to be characterized by its step-wise, molecular mechanisms of carcinogenesis, critical genes implicated in the pathway such as *KRAS*, *PIK3CA, P53,TGF-β*, and DNA mismatch-repair (MMR) genes are actively utilized in the clinical setting to determine individualized therapeutic targets [6]. The *MSI* gene is being used a predictor for favorable response to immunotherapy among advanced colorectal cancer patients [7], and multi-target stool DNA tests are being used in lieu of fecal immunochemical testing for occult blood in screening studies for colorectal cancer with higher sensitivities for detection

of precancerous lesions [8]. In addition, the field of breast oncology has widely implemented the use of biomarkers to treatment response, such as ER, PR, HER-2, and Ki-67, in routine clinical use [9]. Therefore, a similar question can be asked with bladder cancer therapy. When will the use of non-specific and impersonalized histopathologic prognosticators, such as tumor grade, be replaced with individualized, actionable molecular prognosticators as part of the routine standard of care? Clearly, the implementation and integration of precision molecular medicine to the current diagnostic and therapeutic regimens will require a continued concerted effort to not only develop translational approaches to the diagnosis, treatment, and surveillance of bladder cancer, but also a directed, organized, and multi-disciplinary infrastructure to integrate the various and often disjointed translational research activities to a focused and clearly defined set of goals. Other factors which have hampered the rapid progress from bench to bedside in bladder cancer include the emerging complexities in its molecular pathogenesis, such as tumor heterogeneity, along with the multiple biological pathways that lead to tumor initiation and progression, such as loss of heterozygosity, epithelial to mesenchymal transitions, gain of function mutations, and involvement of cancer stem cells, and the field effect of bladder cancer [3]. Despite the inherent complexity, such pathways also provide tremendous opportunity for developing targeted approaches for the treatment of bladder cancer in all its stages. In order to understand how to incorporate precision molecular medicine in bladder cancer treatment, in this chapter, we will provide an overview on current treatment paradigms in non-muscle invasive and muscle-invasive bladder cancer, and discuss opportunities for integration of personalized molecular approaches to standard clinical care.

Current Diagnosis & Staging Paradigms in Bladder Cancer

The vast majority of bladder cancer is detected after patients present with hematuria. Microscopic hematuria, defined as ≥3 red blood cells per high power field on a re-suspended urine sample sediment visualized on high-magnification microscopy, is present in 2–10% of men and 3–20% of women [10]. There is a wide range of presence of bladder cancer in patients referred for further evaluation with a diagnosis of asymptomatic microscopic hematuria, which has been estimated to be 0.5–5% of patients and up to 7–20% patients with other high risk features [11–13]. Gross hematuria, on the other hand, always warrants further workup as it is associated with a high pre-test probability of malignancy or other significant finding, ranging from 15–25% depending on the presence of other risk factors [11, 14, 15].

Initial diagnostic workup entails performing a cystoscopy and imaging of the upper tract and pelvis, usually in the form of a CT abdomen and pelvis with urogram protocol. Cystoscopic evaluation of the bladder alone has a high sensitivity and specificity, ranging from 0.87–0.95 and 0.99–1.0, respectively, along with high positive and negative predictive values depending on the prevalence of the disease in the population. Similarly, CT urography has a sensitivity of 0.93 and specificity

of 99% in diagnosing bladder cancer [16]. In addition, CT imaging is used for detection of local invasion into perivesical tissue, lymph node involvement, and evaluation of distant metastasis. Recent studies have showed reduced ability of CT scans to detect microscopic perivesical invasion or lower clinical stages, but CT can be used to some degree to detect gross perivesical (clinical T3b) disease and higher stages, with an accuracy of 55–92% [17]. MRI is an alternative to CT for local imaging of bladder cancer, and yields excellent tissue and planar detail but lacks spatial resolution. Although earlier studies reported MRI accuracy of 73–96%, this was prior to the widespread use of multi-detector CT scans. Both sensitivities and specificities of detecting pathologic lymph nodes on CT scan is low, due to occurrence of false positives, however, current recommendations suggest that pelvic lymph nodes ≥8 mm or retroperitoneal lymph nodes ≥1 cm should be deemed suspicious for metastatic disease [18, 19]. Other more advanced imaging modalities currently under investigation or in limited use include multi-parametric MRI, particularly with the use of diffusion-weighted sequences, it has been shown to increase the specificity of detection of muscle-invasive tumors from 79% with gadolinium-contrast enhanced imaging to 93% with addition of diffusion-weighted imaging [20]. Although traditional PET-CT with the use of ^{18}F–fluorodeoxyglucose (FDG) has had somewhat of a limited use due to excretion of radiotracer into the bladder and decreased visualization, the use of other radiotracer with less excretion such as ^{11}C–choline has showed promise for both local and distant staging of bladder cancer [21, 22].

There are a multitude of newer technologies that improve overall cystoscopic staging in the diagnosis and treatment of bladder cancer. Two of the most commonly used include blue light cystoscopy, also known as photodynamic diagnosis, and narrow band imaging (NBI) cystoscopy. Blue light cystoscopy is used in conjunction with traditional white light cystoscopy, and has been shown to increase the detection rate of Ta tumors (odds ratio [OR] 4.90, 95% CI 1.94–12.39) and CIS lesions (OR 12.37, 6.34–24.13), along with significantly lowering the recurrence rates for up to 12 months in patients with T1 or CIS lesions (relative risk [RR] 0.70, 95% CI 0.48–1.00; $p = 0.05$) and Ta tumors (RR 0.80, 0.65–0.99; $p = 0.040$) [23]. In a network meta-analysis of 15 randomized controlled trials comparing blue light, NBI, and white light cystoscopy, there was a significant decrease in recurrence rates using either blue-light or NBI cystoscopy compared to white-light alone, but no significant differences in recurrence with blue-light versus NBI cystoscopy were observed [24].

Current Treatment Paradigms in NMIBC

Currently, NIMBC is widely categorized as Ta, T1, or carcinoma in situ (CIS) tumors on pathological review. Although 75% of bladder tumors at presentation fall into the category, the current and prior labels frequently used for these tumors, such as “superficial bladder cancer” or “low-risk bladder cancer” can be misleading. Both clinical experience and recent molecular analysis of NMIBC have revealed

that, despite being included in the same group, there is a wide spectrum and significant differences in overall prognosis of solitary, low grade Ta tumors to more invasive types, namely CIS and T1 disease. The 15-year progression-free survival in patients with low-grade Ta disease is 95% with no risk of succumbing to their disease. On the other hand, those with high-grade Ta tumors had a progression-free survival of 61% and a disease-specific survival of 74%, while patients with T1 disease had a progression-free and disease-specific survivals of 44% and 62%, respectively [25]. In addition, accurate clinical staging of these tumors can often be challenging, and pathological reporting and concordance of results has remained non-uniform, which can lead to both under-staging and over-staging, and thus have significant implications in patient outcomes.

Clinicopathological Risk Stratification of NMIBC

NMIBC is a tremendously heterogeneous disease category. The accurate diagnosis of NMIBC is reliant upon adequate cystoscopic staging and performance of a complete TURBT with removal of all grossly visible tumor to a satisfactory depth that includes muscularis propria. In addition, taking extra steps to rule out the presence of CIS in the bladder and prostatic urethra with all adjunctive available tools, such as the use of blue-light and NBI cystoscopy, and random bladder biopsies is crucial. Moreover, accurate histological diagnosis of the pathology specimen is vital to prevention of under-staging. As a result of the significant heterogeneity among these tumors, there have been multiple efforts in delineating the continuous spectrum of NIMBC into distinct risk categories in order to group together effective therapeutic options. Other groups have published risk tables, nomograms, and artificial neural networks that predict the rates of recurrence and progression on an individualized basis [26–29], which can often be complicated, cumbersome, or time-consuming to use in a busy clinical setting. Although generalized risk stratifications can potentially ease clinical decision-making, especially in the extreme ends of each risk category, it can also add to the confusion with regard to management of less accurately defined categories [30].

There have been a number of professional and international organizations that have put forth risk categories for NMIBC, each with slight variations in definition [31–35]. Some of the most commonly used ones from the American Urological Association and the International Bladder Cancer Group define risk categories on the basis of factors that are associated with increased risk of recurrence and progression, including stage, grade, multi-focality, tumor size, frequency and timing of recurrences, presence of CIS, and other associated high risk factors, including variant histology, lymphovascular invasion, and depth of lamina propria invasion (subcategorizing stage T1) [36, 37]. As a result, NMIBC is divided into low risk, intermediate risk, and high risk groups (Table 5.1). Low risk is generally narrowly defined as a solitary, non-recurrent, low-grade, Ta papillary tumor. The accepted treatment options include complete TURBT followed by a single instillation of

Table 5.1 NMIBC risk stratification

Risk category	Clinicopathologic features	Associated risk factors
Low	Low grade and Ta (non-invasive papillary tumor) and single tumor and no recurrence	None
Intermediate	Low grade and multifocal or recurrent	1. Size (tumor ≥3 cm) 2. Number (>1 tumor present) 3. Timing of recurrence (<1 year since last resection) 4. Frequency of recurrence (>1 per year)
High	High grade or T1 (invasive into lamina propria) or CIS	None

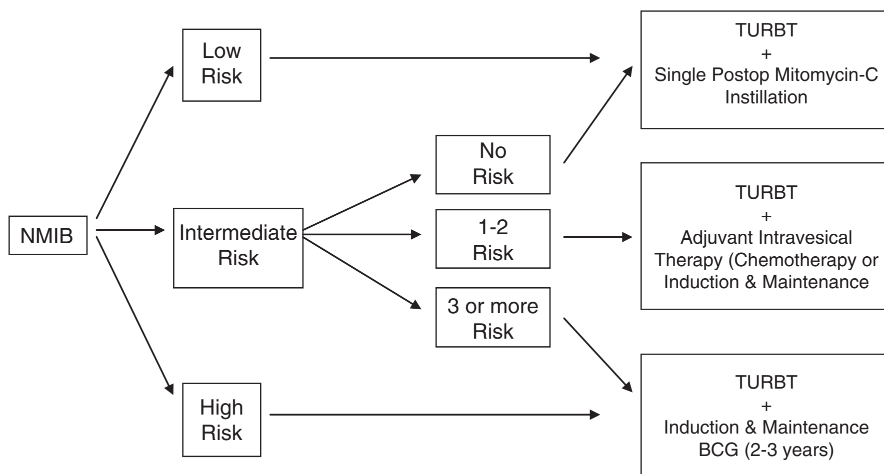

Fig. 5.1 NMIBC risk-adjusted treatment algorithm

post-operative intravesical chemotherapy, such as mitomycin-C, office fulguration, or observation/surveillance. On the other hand, high-risk NMIBC is defined as any high grade, T1, or CIS tumor, with treatment including complete TURBT followed by induction and 2–3 years of maintenance BCG or cystectomy. Intermediate risk NMIBC bladder cancer has been variably defined, but generally includes low grade Ta tumors, that are multifocal or recurrent [30]. Due to the confusion among urologists with regard to selection of the most adequate treatments among this group of patients, a recent review of the literature on intermediate risk disease proposed further sub-categorization by allocating a number of risk factors, including: (1) multifocality, (2) tumor size >3 cm, (3) recurrence earlier than 1 year, or (4) greater than one recurrence per year. Patients with zero risk factors are treated similar to the low risk group. Those with 1–2 risk factors are recommended to undergo complete TURBT followed by adjuvant intravesical therapy (i.e., chemotherapy or induction and 1 year of maintenance BCG). And those with 3 or more risk factors are treated similar to the high risk group [30] (Fig. 5.1).

Opportunities for Integration of Molecular Therapeutics in NMIBC

There is a significant unmet need for integration of molecular markers and therapeutics in the current treatment algorithms for NMIBC. Despite efforts to risk stratify patients based on clinical and histological predictors, current treatment approaches tend to lump many patients into a one-size-fits-all approach, creating the opportunity for both overt-treatment of low-risk progressors and under-treatment of high-risk progressors. There has been a tremendous increase in the wealth of knowledge and insight into the diverse molecular pathways for both muscle-invasive and non-muscle invasive bladder cancers, much of which is the focus of this book and is explained in great detail in other chapters. There is a unique opportunity for real-time monitoring of response to treatment due to relative ease of access to tissue in bladder cancer, and thus, the environment is very ripe for integration of molecular and novel targeted therapies into the current algorithms for NMIBC. As predictive and prognostic markers and the overall genomic landscape for NMIBC are discussed in great detail elsewhere, we will focus on opportunities for integration of current novel therapeutic options for NMIBC in this section.

Opportunities for Molecular Targeted Therapy in NMIBC

The recent publication of The Cancer Genome Atlas (TCGA) data for MIBC has demonstrated that the bladder cancer genome is highly mutated with a high prevalence of intra-tumoral heterogeneity [38]. Similar smaller scale studies on NMIBC show a number of potentially actionable pathways that are distinct from MIBC. Although there are a number of different alterations in genome when comparing both invasive and non-invasive disease, one potential actionable target is the fibroblast growth factor receptor 3 (FGFR3) oncogene, which is frequently mutated in NMIBC, with alterations occurring in 50–70% of cases, particularly in low-grade and stage disease [39–41]. There are multiple mechanisms that lead to aberrant expression of the FGFR3 gene in NMIBC, including point mutations, fusion proteins, isoform switching, and up-regulated expression, all of which increase gene expression and downstream effects [42]. In addition, FGFR3 mutant tumors appear to occur with the same frequency as wild-type tumors, but have lower rates of progression to muscle invasion. A recent study demonstrated 5-year progression-free survival of 91 versus 74% in FGFR3 mutated genes compared to wild-type genes. As a result, the FGFR3 gene could be used as a potential target for decreasing rate of progression in appropriately selected patients. There are a number of different FGFR-specific tyrosine kinase inhibitors currently in Phase I and II clinical trials, with most drugs showing a cytostatic effect on tumor cells [43–45]. Nevertheless, most of these agents have been tested in the setting of advanced solid tumors, and are administered orally. Due to high toxicity profiles, many of these agents would not be appropriate for use in

NMIBC patients, where gold-standard therapies exist with less side effects [46]. One option for circumventing this would be intravesical instillation of FGFR3-inhibitor agents. Such agents could potentially be used in the setting of BCG-refractory or unresponsive disease, or shortly after transurethral resection of low-grade appearing tumors in order to decrease the rate of recurrence or progression [47]. Although topical formulations of this class of drugs currently do not exist, due to high prevalence in NMIBC, this may present in opportunity for integration of molecular targeted therapy into the clinical setting. Another underlying challenge is the development of resistance after continuous exposure to these agents. Understanding the mechanisms of acquired resistance will be crucial to counter-acting its development in the future [46].

Despite the inherent complexities in studying the wide array of prevalent mutations in NMIBC, one potential benefit is the high prevalence of multiple actionable pathways for molecular therapeutic targeting. Preliminary studies examining targetable alterations in NMIBC demonstrate PI3K/mTOR pathway alterations in 37–48%, and CREBBP/EP300 pathway alterations in 26–41% of high-grade NMIBC specimens, both of which are targetable by PI3K/mTOR and HDAC inhibitors, respectively [40, 41]. The difficulty in using these agents lies in determining the sequence of use and prioritizing agents in the setting of multiple co-existing mutations, which is almost always the case with bladder cancer. Although the knowledge for molecular characterization of targetable pathways in many aspects of NMIBC is exponentially on the rise, clinical integration of these findings into existing algorithms will require proper clinical trial design in the setting of collaborative, multi-institutional led studies.

Opportunities for Integration of Immunotherapy in NMIBC

The concept of immunotherapy in NMIBC is nothing new. Despite its initial introduction in the 1970s, to this day, intravesical BCG remains the standard of care treatment for intermediate and high-risk NMIBC and represents one of the most successful immunotherapy stories in cancer treatment [48]. In addition, due to its high mutational rates, bladder cancer is thought to be among one of the more immunogenic cancers in humans [49]. And although the initial response rates for BCG in appropriately selected patients are as high as 70%, due to high recurrence and progression rates in high-grade NMIBC, many patients ultimately succumb to needing alternative strategies for management, the majority of which include extirpative surgery. Therefore, strategies that integrate potential immunotherapeutic agents to allow for bladder-sparing options have long been considered in the treatment of NMIBC.

The use of cytokines as agents to illicit and enhance immunomodulatory effects has previously been studied for NMIBC. Interferon alpha 2b (IFN) has been combined with BCG for use as an intravesical topical agent in both BCG-naïve and BCG-refractory patients with NMIBC. The largest of these was a Phase II, multi-institutional trial including 1007 patients, 46% of which were BCG-failures and had a 2-year recurrence-free rate of 45% with combination IFN and BCG

intravesical treatment [50]. Due to its transient presence in the bladder, IFN has had a limited duration of response. One strategy that has been used to improve the efficacy of IFN is intravesical gene therapy which combines a recombinant IFN adenoviral vector with Syn-3, a clinical surfactant that enhances gene delivery to the urothelium [51]. In a Phase I clinical trial, a total of 17 patients with recurrence after BCG were treated with a single dose of the combination gene therapy agent and various concentrations, there were no dose-limiting toxicities, and high dose-dependent levels of IFN were detected in the urine indicating efficient gene transfer. Among the 14 patients treated at doses of 10^{10} or more particles per ml with detectable urine IFN, 6 (43%) experienced a complete response at 3 months and two remained disease-free at 29.0 and 39.2 months, respectively [52]. A phase II study is recently completed. This and similar gene therapies could potentially be integrated into the current treatment algorithms for NMIBC at the level of first- or second-line therapy for BCG-failure patients. Further study is required to ascertain the level of response and appropriate dosages for these patients.

Immune checkpoint inhibition blockade is another emerging therapy that has shown promising results in many other types of cancer, including renal cell carcinoma, melanoma, and non-small cell lung cancer [53–55]. The premise of this strategy involves administration of monoclonal antibodies that specifically block inhibitory cell surface receptors on the antigen-presenting cell or effector T-cell that ordinarily lead to quiescence of the immune reaction and decreased T-cell proliferation. Although a number of potential molecular targets are currently under investigation, initial trials have focused on inhibition of CTLA-4, a T-cell co-receptor that interacts with the B7 family of cell surface receptors on the antigen-presenting cell, primarily in the lymphatic tissue, along with inhibition of PD-1 or PD-L1 co-receptors on the cytotoxic T-cell and tumor cell, respectively, that are primarily present in the tumor microenvironment [53, 55]. The vast majority of immune checkpoint blockade trials in the bladder cancer domain were initially conducted as second-line therapy for patients with advanced, metastatic disease unresponsive to standard therapies or as first-line therapy in combination with standard regimens in metastatic patients. However, two recent trials are currently being conducted in patients with non-metastatic bladder cancer. NCT02324582 is a Phase I, single-arm trial studying the safety and efficacy of pembrolizumab, an anti-PD-1 monoclonal antibody, co-administered with standard intravesical BCG therapy in patients with high-risk NMIBC who have experienced BCG failure. Secondary endpoints include complete response rate and quality of life measures. Another Phase II, single-arm, open-label study of atezolizumab, an anti-PD-L-1 monoclonal antibody, is currently recruiting in patients with either high-risk NMIBC that are BCG-refractory or patients with MIBC who are surgical candidates and either refuse or are ineligible to undergo neoadjuvant chemotherapy (NCT02451423). In addition to the above-mentioned pathways, there are a number of other immune checkpoint pathways such as LAG-3, TIM-3, TIGIT and several others that may potentially be exploited, however, preliminary data in the pre-clinical setting suggests that T-cell immune checkpoint inhibition likely involves multiple pathways, some of which are non-overlapping [56]. This would imply that there may be a need for utilization of

multiple checkpoint inhibitors to derive adequate clinical benefit. In addition, the toxicity profile of many currently used immune checkpoint inhibitors is well known from studies conducted in patients with advanced disease. The overall side effect profile and toxicity appear to be favorable compared to standard therapies in this setting; however, administration of these agents in patients with NMIBC who generally have favorable prognosis and minimal treatment-related side effects may prove to be challenging. Given the vast array of possible immunotherapy combinations and potential for new emerging treatments in NMIBC, particularly in patients with BCG-refractory disease, further study and large-scale, multi-center collaborations are necessary to better identify patients with sensitivity to certain immune checkpoint blocking agents and determinants of immune checkpoint biomarker expression before this is implemented in standard clinical practice.

Risk-Adjusted Treatment Paradigms in MIBC

Approximately 25% of patients who initially present with bladder cancer will have muscle-invasive disease (T2–T4) (Table 5.2). As the natural history of these patients is associated with almost universal poor outcomes, aggressive treatment is indicated [57]. The standard of care treatment for MIBC is radical cystectomy with bilateral pelvic lymphadenectomy. Since the procedure was first refined by Marshall and Whitmore in the mid-1950s, there has been significant improvement in overall outcomes with decreased morbidity due to improved surgical technique and enhanced perioperative care [58]. Nevertheless, radical cystectomy alone still results in unacceptably high recurrence rates, mostly due to distant disease. Overall 5-year mortality rates for patients with MIBC are reported to be up to 50% after cystectomy alone [59, 60]. Thus, the concept of MIBC as a truly systemic disease resulted in the use of neoadjuvant chemotherapy in an attempt to eliminate the presence of micrometastatic disease prior to undergoing surgical extirpation. Two large Phase III randomized prospective clinical trials established the evidence to support this practice. The EORTC study used a neoadjuvant chemotherapy regimen of methotrexate, vinblastine, and cisplatin (MVC) which resulted in a 16% reduction in the risk of death (HR, 0.84; 95%CI, 0.72–0.99; $p = 0.037$) in patients undergoing neoadjuvant chemotherapy followed by radical cystectomy compared to those undergoing surgery alone [61].

Table 5.2 MIBC risk-adjusted treatment

Risk category	Clinicopathologic features	Treatment
Low	MIBC with no high risk features	Radical cystectomy, bilateral pelvic lymph node dissection
High	• Hydronephrosis • Lymphovascular invasion • Clinical T3b or greater • Variant histology	Neoadjuvant chemotherapy followed by radical cystectomy, bilateral pelvic lymph node dissection

Another trial by SWOG in the US after randomly assigning patients to neoadjuvant chemotherapy with 3 cycles of methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) followed by cystectomy versus surgery alone demonstrated similar results. Importantly, 38% of patients undergoing NAC had pathologic down-grading of disease to pT0 versus 15% of patients with surgery alone, and those with pT0 disease had overall significantly improved prognosis with 5-year disease-specific survival of 85% [62]. A comprehensive meta-analysis updating the overall effect of neoadjuvant chemotherapy in patients with MIBC demonstrated an absolute overall survival improvement of 5% and a disease-free survival improvement of 9% at 5 years [63].

Although the addition of neoadjuvant chemotherapy has clearly improved outcomes, variable response rates among patients along with overall relatively modest absolute benefits have led to efforts to improve risk stratification among patients eligible to receive NAC. In addition, grade 3 and 4 toxicities have been reported in the range of 35–40% in both SWOG and EORTC studies [61, 62]. It has become increasingly clear there are a subset of patients that may not benefit from NAC, which only results in patients enduring the multitude of potential side effects with no added benefit, and a potential delay in definitive treatment. Due to the lack of available molecular markers that could be used as surrogates for response, there have been a number of studies evaluating clinicopathologic methods of risk stratification. Initial subgroup analysis of the SWOG and other studies revealed that the most dramatic improvement in median survival in patients undergoing NAC occurred in those with clinical T3 or T4 disease [61, 62]. This finding led to further risk stratification of patients with MIBC into high risk and low risk groups, with high risk defined as patients with ≥cT3b disease, presence of hydroureteronephrosis, lymphovascular invasion (LVI), or variant histology, such as squamous, sarcomatoid, neuroendocrine, etc. components. In a study of 297 patients investigating clinical and pathological risk factors in MIBC, high risk patients were found to have decreased overall survival of 47 versus 64.8% in the low risk group, along with decreased disease-specific survival (64.3 versus 83.5%) and progression-free survival (62 vs. 84.1%) probabilities in the high versus low risk groups, respectively [64]. These risk groups were recently externally validated in a separate cohort of 449 patients, where 5-year cancer-specific mortality-free rates of 64.4 versus 77.4% in the high versus low risk groups were reported [65]. These studies confirm that preoperative clinicopathologic risk factors can stratify patients with MIBC based on survival probabilities, and therefore, those with lower survival are likely to benefit more from NAC.

Clinical Applications of Molecular Pathways in the Treatment of MIBC

There is a significant unmet need for integration of precision molecular medicine in the current treatment regimens for MIBC. Much of the challenge lies in identifying appropriate targets out of the milieu of genetic and epigenetic

alterations that exist in MIBC and matching patients with the correct genetic signatures to the most appropriate agents with the highest likelihood of response. The presence of intra-tumoral heterogeneity and the vast array of possible mutations add to the complexity and biological diversity of bladder cancer [66]. Nevertheless, as a result of next-generation sequencing and high-throughput genetic techniques, there has been a significant increase in the understanding of the molecular pathways that lead to oncogenesis in bladder cancer and hence, insights into personalization of therapeutic approaches of this tremendously heterogeneous disease.

A molecular signature for MIBC has been discovered and confirmed by three independent groups that have recently characterized intrinsic subtypes that bear remarkable resemblance to the molecular characterization of breast cancer, which is discussed in detail in other chapters [67]. Researchers from University of North Carolina, University of Texas MD Anderson Cancer Center, and the University of Lund have, in parallel, categorized MIBC into overall basal and luminal subtypes, findings which were further categorized by The Cancer Atlas Genome project [38, 67–69]. Luminal tumors showed active PPARγ transcription and were highly enriched with activating FGFR3 mutations, demonstrating a potential for FGFR inhibitor responsiveness. In addition, a subset of luminal cancers has been identified as p53-like, which are characterized by high levels of stromal fibroblast infiltration. On the other hand, basal tumors were highly enriched with p63 activation and squamous differentiation [68]. From a clinical standpoint, when these intrinsic subtypes were correlated to disease-specific outcomes, it became evident that basal tumors were associated with more locally advanced stage and metastatic disease at initial presentation, and had poorer overall and disease-specific survival compared to luminal tumors [38, 68]. Moreover, while more than half of both luminal and basal tumors demonstrated at least partial response to neoadjuvant chemotherapy, p53-like subtypes were resistant to current standard platinum-based neoadjuvant chemotherapy [38, 68]. The clinical implications of these early data suggest that perhaps the addition of molecular subtyping to current clinical paradigms can lead to improved risk-stratification of neoadjuvant chemotherapy responders while sparing non-responders the potential side effects and delayed definitive surgery associated with chemotherapy. Alternatively, patients with p53-like tumors should be treated with other neoadjuvant regimens, such as FGFR inhibitors or other systemic targeted treatments. In addition, whole-exome and next-generation sequencing of platinum-based NAC patients showed that ERCC2, a nucleotide excision repair gene, and ERBB2/HER2, a receptor tyrosine kinase oncogene, were the only significantly mutated genes enriched in the cisplatin responders compared to non-responders, which were also confirmed by in-vivo studies [70, 71]. Continuous research efforts in this area are necessary to eventually replace the current clinical paradigms used for identification of patients with high-risk MIBC with molecular risk-stratifiers that translates directly clinical outcomes predictions.

NAC Model for Precision Therapy in Bladder Cancer

It is clear that the standard clinical paradigms for assigning which patients with MIBC benefit the most from NAC is lacking. Current algorithms for clinicopathologic risk stratification still remain a relatively unsophisticated method to predict clinical benefit. As NAC in its current forms has both a modest response and it significantly underutilized, a new paradigm for improved categorization of patients is desperately needed. MIBC is a potentially optimal condition in which to adapt neoadjuvant treatment paradigms. The reasons for this are multiple. First and foremost, a platform to assess response to neoadjuvant therapy is necessary. Fortunately, biopsy of an abundant amount of tissue in the pre-chemotherapy and post-chemotherapy settings is readily feasible through both transurethral resection and post-chemotherapy cystectomy. Molecular characterization of pre-chemotherapy tissue specimens may direct whether patients may benefit from platinum-based NAC, for example by incorporating molecular subtyping of MIBC to assess for the presence of p53-like tumors or certain individual genetic mutations [68, 71]. In addition, "real-time" analysis of response to tissue in the post-chemotherapy setting is a possibility in the NAC paradigm, as shown by using immunohistochemistry to measure drug response by assessing the presence of Ki-67, a common marker of proliferation. Another advantage of implementing a neoadjuvant paradigm in MIBC is the ability to use a readily measurable clinicopathologic marker as a surrogate for true complete response. Multiple prior studies have shown achieving pT0N0 disease after NAC is associated with a 5-year survival of over 85% [61, 62, 72], which can potentially serve as a foundation for comparison with future neoadjuvant regimens. Moreover, any residual bladder tumor remaining after NAC will by definition be chemo-resistant, and represents a significant opportunity for molecular profiling through whole-exome sequencing and transcriptome arrays to identify specific drivers for resistance and tailor appropriate targeted therapies. Similar strategies can be extended out the metastatic setting.

Although there are multiple advantages to applying the neoadjuvant paradigm to the current standard clinical algorithms for MIBC, there are also significant drawbacks. As bladder cancer is a tremendously diverse disease, inter-tumoral and intra-tumoral heterogeneity with both TURBT and radical cystectomy can be a significant source of confounding results when testing for molecular signatures. In addition, at least during initial implementation, there may be a significant cost associated with high-throughput genomic processing of each individual patient specimen multiple times throughout the process in order to derive the specific molecular characterizations necessary to direct precision therapy. Nevertheless, as the treatment of bladder cancer steadily progresses towards translational oncology and precision molecular medicine, the incorporation of comprehensive individualized treatment algorithms into existing clinical paradigms will not only serve to improve overall patient outcomes, but also avoid unnecessary and costly side effects associated with non-specific, universal therapies.

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Chapter 6
Requisite for Collection and Distribution of Tissue and Fluid Specimens for Molecular Diagnostics and Discovery in Bladder Cancer

Adrie van Bokhoven and M. Scott Lucia

Introduction

There is increasing demand for molecular tools to aid in the clinical diagnosis and management of patients with bladder cancer. Beside limited use of fluorescence in situ hybridization (FISH) analysis as a diagnostic tool using urine cytology specimens, there are currently no routinely utilized molecular methods for bladder cancer diagnostics in clinical practice. However, as increasingly has been the case for tumors from other organ sites, there are a large number of molecular biomarkers for bladder cancer in various stages of development. These new markers cover the full spectrum from DNA mutations to mRNA and miRNA expression profiles. In addition to tissue obtained from cystectomy, the accessibility of the bladder offers the ability to collect tissue by biopsy or transurethral resection of lesional material. There are also minimally-invasive specimen collection options to obtain diagnostic specimens such as urine and bladder washings. Many factors from procurement to final assay analysis can affect the outcome of a molecular diagnostic test. While molecular testing methods are constantly updated and improved to push the boundaries allowing for a larger range of routinely collected clinical specimens suitable for testing, there is increasing need to optimize procurement and processing of specimens to improve preservation to make them more reliably analyzable for a wider range of molecular diagnostic test procedures. Compatibility with the reality of routine clinical practice has to be balanced with diagnostic needs and benefits. In this chapter we will describe specimen collection and processing methods currently used in clinical practice, with its benefits and caveats, while also describing more

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advanced procurement and processing methods for improved preservation of specimens to make them assessable for a wider range of advanced molecular diagnostic tests that find its way into clinical practice.

Specimen Sources and Procurement Methods

Specimens can be obtained through a variety of procurement methods ranging from non- or minimally-invasive to full surgical resection procedures. Table 6.1 outlines the common procedures and the possible specimen types for which molecular analysis is possible.

Most of the tissue types listed in Table 6.1 can be used in a wide variety of DNA, mRNA, and miRNA analyses.

The primary goal for which a specimen is collected varies depending on the state of the disease. For instance, for patients at risk for or suspected of having bladder cancer the main reason for obtaining specimens is to aid in the diagnosis, e.g. taking a urine specimen for cytological analysis or taking a biopsy from a suspicious lesion in the bladder. After the diagnosis of bladder cancer is made, procedures are usually performed for curative intent. However, these procedures also provide valuable tissue sources for tests that can add prognostic value to predict treatment outcome and guide treatment modalities. When dealing with a biological specimen that is procured or targeted for diagnostic use, there are several factors that need to be taken into account. For most tissues and diagnostic methods, the

Table 6.1 Overview of procedures and specimen types

<table>
<tr><th>Tissue type</th><th>Method</th><th>Routinely obtained specimen type</th></tr>
<tr><td rowspan="3">Bladder tissue</td><td rowspan="2">Cytology</td><td>FFPE</td></tr>
<tr><td>Ethanol-based fixative</td></tr>
<tr><td>Brushing, biopsy, TURBT, cystectomy</td><td>FFPE</td></tr>
<tr><td>Metastatic tissue</td><td>Biopsy, surgery, lymph node dissection</td><td>FFPE</td></tr>
<tr><td rowspan="3">Urine</td><td rowspan="3">Voided (VB1, VB2), catheterized</td><td>Ethanol-based fixative</td></tr>
<tr><td>Exfoliated cells</td></tr>
<tr><td>Isolated fractions: DNA, RNA</td></tr>
<tr><td rowspan="3">Bladder washing</td><td rowspan="3">Active sampling, barbotage</td><td>Ethanol-based fixative</td></tr>
<tr><td>Exfoliated cells</td></tr>
<tr><td>Isolated fractions: DNA, RNA</td></tr>
<tr><td rowspan="5">Blood (liquid biopsy)</td><td rowspan="5">Venipuncture</td><td>Circulating tumor cells</td></tr>
<tr><td>Cell-free circulating tumor DNA (ctDNA)</td></tr>
<tr><td>Whole blood</td></tr>
<tr><td>Isolated DNA/RNA</td></tr>
<tr><td>Normal control DNA</td></tr>
</table>

overarching objective is to obtain a specimen in which the analyte is as closely reflective of its state when it was in the patient's body. Preserving the integrity of the tissue morphology as best as possible is the primary objective for tissue specimens that will be used for standard pathological evaluation. Similarly, for molecular analysis the analytes (DNA/RNA) should be preserved as best as possible in the state they were in the patient's body. The goal for which a specimen is procured together with the method of procurement and preservation will determine what diagnostic methods can be successfully utilized to provide reliable and reproducible results.

The three factors that have the greatest influence on the specimen quality and its utility for specific clinical diagnostic tests are: time from removal of the specimen from the body to testing, temperature, and preservation method. As discussed below, these factors have different impacts on the various specimen types (cystectomy, biopsy, urine) that are to be tested. Unfortunately, not all of these factors are equally controllable in clinical practice, and there are wide ranging differences in how individual clinical practices are organized and equipped to deal with procurement and handling of diagnostic and surgical specimens for molecular analysis.

Molecular Diagnostic Analysis Options

While new and emerging molecular analysis techniques are promising powerful new tools to aid the clinician in the management of patients diagnosed with bladder cancer, they are also providing some unique challenges to the standard handling and processing methods that have been used in pathology. There are numerous analysis methods to identify differences between normal tissue and tumor tissue. These range from analysis of chromosomal abnormalities, mutation and methylation analysis of DNA, to mRNA or miRNA expression analysis of a single gene or profiling of large sets of genes. While many of these techniques can perform very well in the research setting to obtain insight into the tumor biology, most of them are not very well suited to be implemented in clinical practice [1–5]. For example, many techniques require fresh or frozen tissue for optimal assay performance, while it is to be expected that processing of tissue into formalin-fixed paraffin-embedded (FFPE) samples will continue to be the mainstay of pathological practice for routine clinical care. Most molecular testing laboratories prefer to be provided with FFPE tissue blocks for testing since they are intimately familiar with the testing methods and can make a better determination whether tumor cellularity requires additional enrichment by macro- or microdissection (see below). In addition, most molecular laboratories are setup better to perform sectioning in way that prevents or minimizes cross-contamination between cases. In many cases molecular analysis will be done in a research environment on patient material specifically procured for research purposes. These results should not be used for patient care if specimens were not collected, handled, stored, and tested under College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA) accredited

conditions. In most of these cases for the finding to be used in patient care the test will have to be repeated on material handled and the test run in a CAP/CLIA certified environment.

Solid Tissue

As outlined in Table 6.1, bladder tumor tissue can be obtained from several different procedures and sources including biopsy material obtained during cystoscopy, transurethral resection of the bladder tumor (TURBT), or complete removal of the bladder by cystectomy. While increasingly there is a need for fresh and frozen tissue for molecular analysis, current routine pathological practice in most institutions is that for each of these methods, resected material is fixed in formalin and processed into FFPE tissue sections or "blocks."

While smaller tissue fragments such as those obtained from a biopsy or TURBT are usually immediately transferred to formalin after leaving the body in the procedure room or surgical suite, larger specimens from cystectomy and lymph node dissection might require additional handling and dissection in the pathology suite to guarantee proper fixation of the resected tissue. In cases where the bladder is still intact just submerging the whole specimens in formalin would not be optimal since passive transfer of fixative to the bladder mucosa through the urethra will be slow and allow air pockets that get caught internally to prevent direct contact of the tissue with the formalin solution. One option is to inject formalin with a syringe into the bladder through the urethra. Through this procedure the bladder will be inflated after which the urethra is clamped to prevent the bladder from deflating and formalin flowing out of the urethra. After filing the bladder, the complete specimen is put in a container large enough so the whole specimen is submerged in formalin. The volume of formalin should preferably be 10–20 times the volume of the tissue. This procedure can limit the amount of formalin available to fix the interior of the bladder since there is no direct contact between the relative small amount of formalin inside the bladder with the excess formalin around the exterior of the bladder. Alternatively, and preferably, the bladder specimen is opened anteriorly and the bladder walls pinned (Fig. 6.1a) or propped (Fig. 6.1b) open to render the inside of the bladder assessable for evaluation and fixation with formalin.

As mentioned, timing of the processes of procurement and fixation is an important factor in tissue preservation. Warm ischemic time is defined as the time between clamping off the blood supply to the organ and when the dissected organ leaves the body (out of body time). Cold ischemic time is the time between the out of body time and when the tissue is put in formalin or preserved in another manner. Unfortunately, both warm and cold ischemic times can be highly variable and both are important factors in tissue preservation. During both times the tissue is still biologically active and may drift from its normal biology. Clamping of the blood supply can lead to hypoxic conditions which can lead to tissue degradation. In addition, cells and tissue are put under stress which can lead to a tissue response

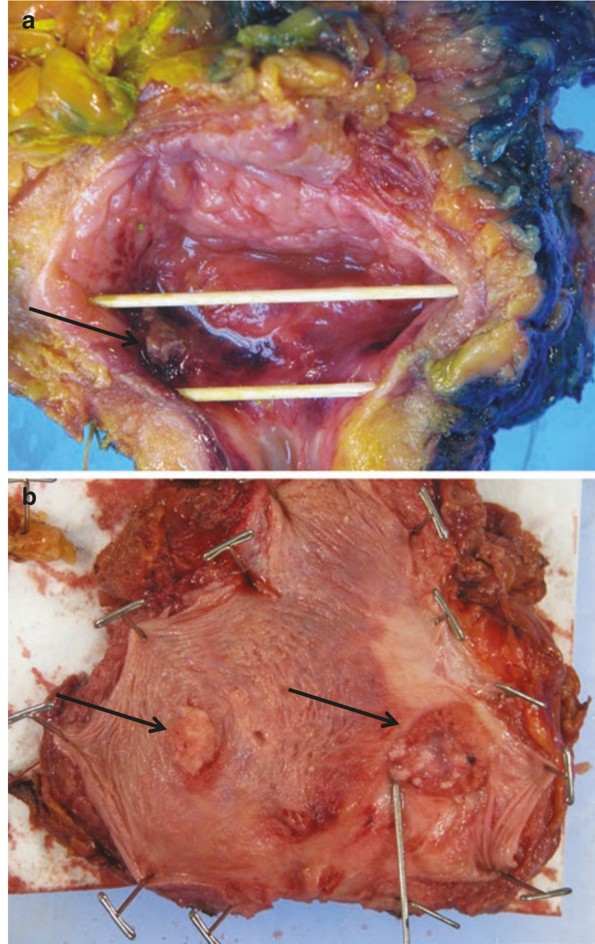

Fig. 6.1 Radical cystectomy specimens anteriorly opened and propped (**a**) or pinned out (**b**) for submersion into formalin bath. *Arrows* denote foci of carcinoma

and changes to the molecular targets. Changes in RNA expression can be rapid and can lead to irreproducible analysis results or can be wrongly attributed to tumor characteristics [6–10]. Unfortunately, warm ischemic times are not very controllable and can vary widely since they are dependent on many factors including patient condition, body habitus, surgical procedure type and facilities, and surgical skill. Clamping times and out of body times are also not routinely recorded. To keep cold ischemic time as short as possible, tissue should be fixed in formalin as soon as possible after the material is removed from the patient. Currently, the time tissue is transferred to formalin is often not recorded. In addition, the fixation time, the time that the tissue is submerged in formalin and the fixation takes place, can be highly variable based on procedure, size of the specimen, time of the day, day of the week, and workload of the pathology team. The two factors driving formalin fixation are penetration, the ability of the solution to diffuse into the tissue, and the ability of the fixative to complete the initial molecular crosslinking. Initial

crosslinking of molecules in tissue is reversible and forms the basis of antigen retrieval methods [11, 12]. While under-fixation can lead to misleading and irreproducible molecular analysis results, longer fixation times and permanent storage in formalin leads to irreversible crosslinking making the tissue less suitable or unusable for immunohistochemical and molecular analysis [13]. Thickness of the tissue sample in the range of 2–5 mm is recommended so that complete penetration occurs within 24 h at room temperature. Small specimens like needle biopsies are routinely left in formalin for 6–24 h but larger specimens may require longer times to allow for complete penetration and fixation. As penetration of the fixative occurs relatively slowly, the outermost layers of the tissue specimen might experience a longer fixation time than innermost layers of the tissue. This can lead to increased degradation on the innermost portions of the tissue while the outer layers might get over-fixed [14]. In addition, as fixation progresses in the outermost tissue layer, it has the potential to further delay fixation of the inner layers by interfering with diffusion of formalin [15]. This fixation and/or degradation gradient in the tissue can lead to variable and irreproducible results of downstream analysis in different layers in the tissue [16]. Unfortunately, in most cases the steps within the process of the material leaving the body to a fully processed FFPE tissue block are poorly tracked making it difficult to accurately assess the impact of warm and cold ischemic and fixation times.

Implementing changes in this longstanding process have been difficult but are starting to be driven by requirements of some newer tests that have been shown to be highly valuable to patient care. The best example is HER2 immunohistochemistry testing for breast cancer that has been successfully implemented into routine practice over the last few years [17]. Her2/neu receptor expression by immunohistochemistry is used to identify tumors that are candidates for trastuzumab, a Her2-targeted therapeutic in breast cancer. To be able to correctly interpret the test for HER2, there are several requirements under CAP guidelines in the handling and processing of breast cancer tissue into FFPE that need to be fulfilled. Tissue thickness of material to be fixed in formalin cannot be more than 5 mm and fixation time has to be no less than 6 h and no more than 72 h. Cold ischemic times are kept under 60 min requiring recording of out of body times and prompt transfer to pathology for gross dissection and fixation. Implementing and complying with these guidelines requires tight communication and collaboration between the surgical and pathology teams but has been successfully accomplished. As an additional benefit of implementing these guidelines, the specimens that are handled under these parameters are also better and more consistently preserved for molecular analysis. Recently, the programmed death-ligand 1 (PD-L1) inhibitor atezolizumab received U.S. Food and Drug Administration (FDA) approval for the treatment of patients with advanced urothelial carcinoma that failed or refused standard of care therapy [18]. With this new treatment option there is also a need for the companion diagnostic of the immunohistochemical detection of PD-L1 to identify the patient group that could benefit from atezolizumab treatment. PD-L1 guidelines for tissue handling and preservation are identical to HER2 in breast. Implementation of this test is ongoing and represents a good example how clinical

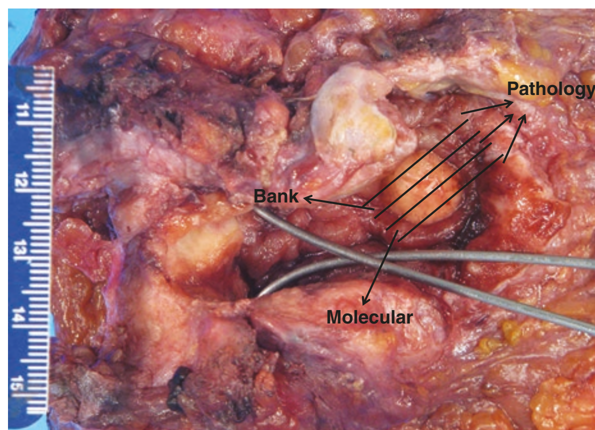

Fig. 6.2 Radical cystectomy specimen opened to reveal mass in *upper right*. Serial sections of the mass can be taken and processed for pathology, molecular analysis, and banking as indicated

need and clear patient benefit can drive changes in routine and established procedures. Based on this we will hopefully see a change in how bladder specimens are routinely handled leading to improved tissue preservation to enable implementation of a wider range of molecular diagnostics. For small tissue fragments like biopsies and TURBT specimens, these guidelines can easily be followed. However, resection of larger tissue fragments from partial or full cystectomy will require adjustments. We envision a better standardization of the process that, when successfully implemented, can be very beneficial to advance molecular analysis as well as research. This will require fast evaluation and resection of tumor areas in the surgical pathology suite to keep cold ischemic times as short as possible. Tumor areas will need to be dissected and the proper areas need to be taken to satisfy pathological and molecular evaluation needs as well as potentially procuring fresh or frozen tissue for tissue biobanking (Fig. 6.2).

The need for standardization of tissue preservation for the full range of resected specimens is important due to the fact that each of the different specimens from diagnostic biopsy to TURBT and cystectomy is potentially needed and used for molecular diagnostics. Biopsy materials are often limited in size and tumor content so care should be taken to preserve material as much as possible to be able to do additional testing on the material.

While TURBT procedures usually result in larger tissue fragments and more tumor material than biopsies, results can be highly variable. As shown in Fig. 6.3, only parts of the collected tissue fragments contain viable tumor material. It is also difficult to obtain fresh-frozen tissue from TURBT without potentially effecting pathological analysis. Cystectomy has traditionally provided the best option to acquire larger amounts of tumor material. Figure 6.4 shows a number of examples of sections taken from FFPE blocks from cystectomy specimens with variable amounts of tumor as delineated on the resultant hematoxylin and eosin stain (H&E) stained slides.

The pathologist is tasked with identifying the appropriate specimen and isolating intact tumor cell in a manner that is most representable of the lesion and best

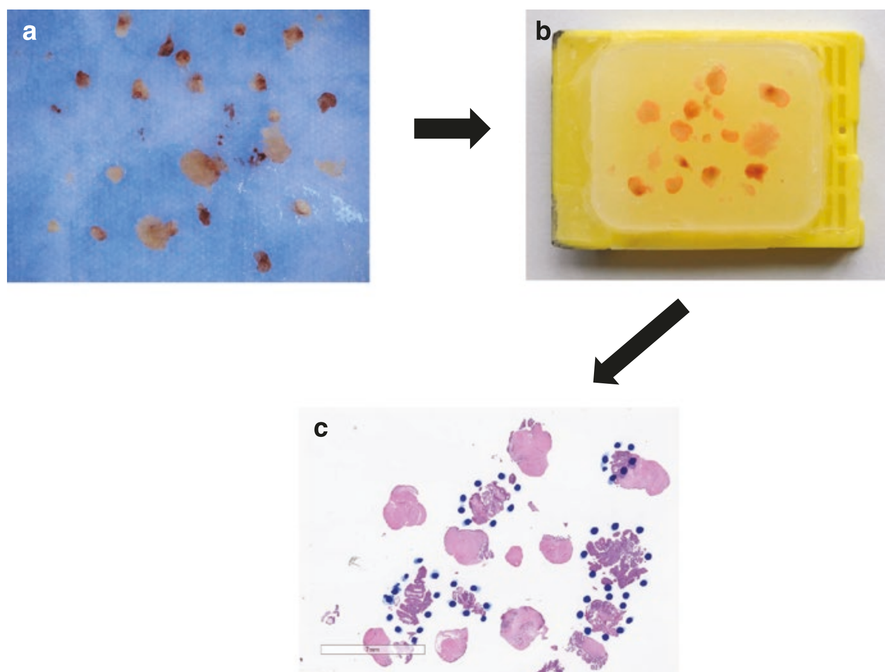

Fig. 6.3 TURBT specimen as placed upon a tissue pad at grossing (**a**), processed into a paraffin block (**b**) and sectioned and stained with H&E (**c**). *Dotted areas* on (**c**) indicate intact tumor

suited to be used for molecular testing. Such "preanalytical processing" includes evaluation of specimen quantity and tumor cellularity, determined by the ratio of tumor cells to non-tumor cells in the sample or region of interest. Even though a particular specimen may contain a large portion of "lesional" material does not necessarily mean that it contains high tumor cellularity. For example, non-tumor cells such as stromal or inflammatory cells may make up a large relative proportion of the total cell nuclei within the lesion thereby diluting the amount of DNA derived from the tumor cells (Fig. 6.5). This could lead to false negative results from a test that has a relatively weak analytic sensitivity. Thus, depending on the molecular analysis technique and tumor cellularity, tumor enrichment might be needed through macro- or microdissection. These processing steps require that the pathologist identify areas of the tissue section containing the highest lesional cellularity and quality under the microscope and then scraping with a scalpel or needle the areas of interest from thin sections on counterstained or matched unstained slides. Such preanalytical processing is best performed at the molecular testing facility since, with increasing demand on tissue specimens for additional molecular testing, this may place increased burden on the local pathologists. Whereas releasing a series of precut unstained slides from the pathology lab to the testing facility is acceptable, release of the most representative FFPE block is preferable and should be done if possible.

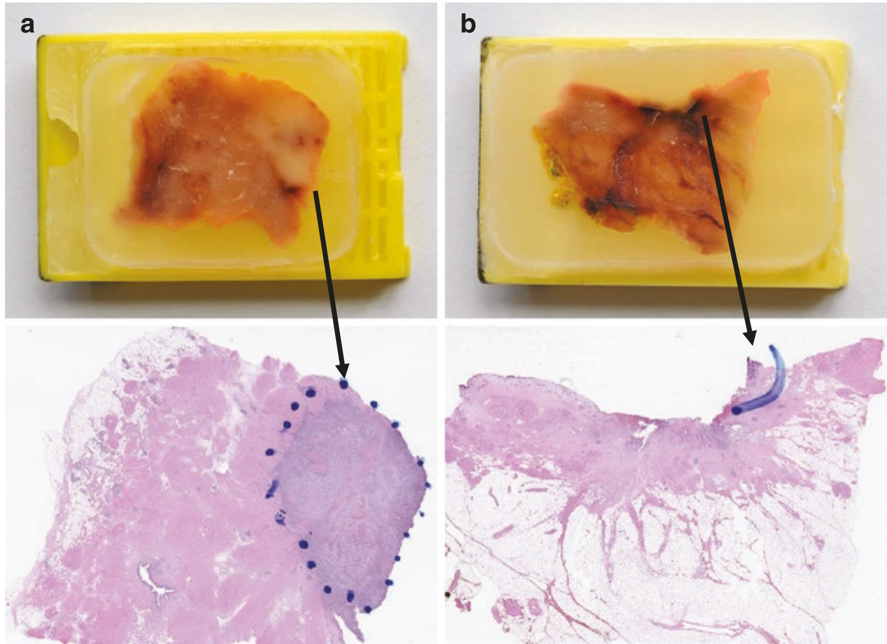

Fig. 6.4 FFPE blocks (*upper panels*) and associated H&E stained slides (*lower panels*) taken from two cystectomy specimens with different amounts of tumor. Panel **a** shows a large nodule of tumor that contains a high proportion of infiltrating lymphocytes in addition to tumor cells (see Fig. 6.5). Panel **b** shows only a small residual focus of intact tumor (denoted by *blue*) after prior TURBT that is not readily visible on the block

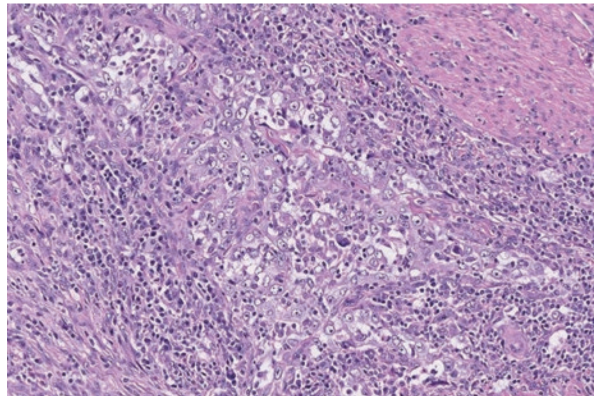

Fig. 6.5 Section of the urothelial carcinoma from Fig. 6.4a with marked infiltration by inflammatory cells (H&E, 200×). The tumor cellularity is low compared with the number of infiltrating inflammatory cells

With neo-adjuvant treatment becoming the standard of care, in many cases cystectomy specimens may contain minimal or no residual tumor. This can also be the case in cystectomy specimens after TURBT as shown in Fig. 6.6. This stresses again the need for standardized handling of all resected tissue since the pre-surgical biopsy or TURBT tissue will potentially be the only available material for ancillary

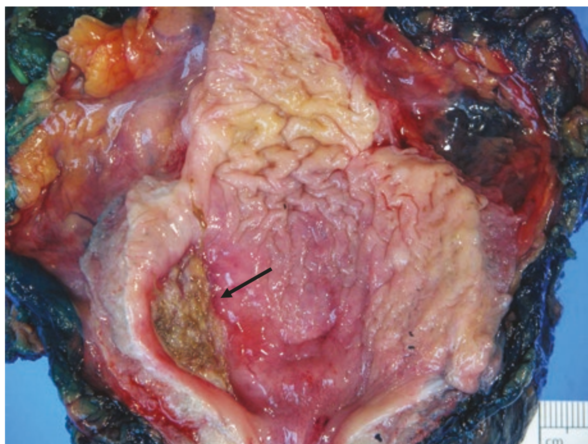

Fig. 6.6 Radical cystectomy specimen performed following previous resection by TURBT. The resection site with fulguration change is denoted by *arrow*. No residual tumor was identified microscopically

molecular testing. For the pathological examination of regional lymph nodes for cancer metastasis, most centers routinely submit all lymph node tissue for formalin fixation and paraffin embedding. The current methodology provides little option to procure materials suited for molecular analysis without microdissection of FFPE sections. Standardizing cold ischemic and fixation times as described above will already be an improvement. Further improvement can be made by dividing lymph nodes in the pathology suite to obtain separate areas of each node for pathological assessment and molecular studies. This is unlikely to impact pathological examination while potentially improving the prognostic value of lymph node dissection through adjunctive molecular analysis. The ability of processing lymph nodes in this manner for molecular analysis has yet to be validated.

Body Fluids: Urine and Blood

Hematuria detected by discoloring of the urine or urinalysis often provides the first indication of urinary tract disease and possible bladder malignancy. Diagnosis of hematuria will often lead to follow-up of the patient with additional diagnostic tests including urine cytology. Urine cytology is based on the fact that bladder cells, including tumor cells, exfoliate into the urine and can be collected and analyzed under the microscope. While urine cytology is successful in diagnosing high-grade tumors and carcinoma in situ (CIS), its sensitivity to detect low-grade non-invasive tumors is much lower [19]. Usually the second morning's voiding is used for cytology. The first morning urine should not be used because cells sitting in urine overnight can become damaged and difficult to analyze. Sometimes urine of three consecutive days is used for analysis to increase sensitivity and specificity. If urine is not processed within 1–2 h after collection, the addition of an ethanol/polyethylene glycol-based fixative such as Saccomanno's is recommended to preserve the exfoliated cells. Bladder washings or barbotage specimens can be used and usually

provide better results since these specimens usually have a higher concentration of exfoliated cells that are better preserved than in voided urine. As mentioned sensitivity of urine cytology is relatively low, but fluoresce in situ hybridization (FISH) has been shown to increase sensitivity and can provide results on as little as 25 cells [20]. The FDA approved UroVision™ Bladder Cancer Kit is used to detect aneuploidy for chromosomes 3, 7, 17 and loss of the 9p21 locus by FISH as an indication of the presence of bladder cancer cells. This test can be of value in the initial diagnosis of bladder cancer and aid in monitoring recurrence. Standard voided urine specimens can be used for this test.

In contrast to solid tissue testing where the preanalytical processing is done by a pathologist, preanalytical testing of urine usually begins in the clinic, ward, or clinical lab. Directly after collection, urine should be mixed 2:1 (v/v) with preservative (Carbowax or PreservCyt). The sample is preferably refrigerated as soon as possible after mixing with the fixative and shipped on cold packs to the testing site, although shipping at temperatures up to 25 °C is permitted. Specimens can be processed into a fixed cell pellet within 72 h after collection. Preservation of cells by fixation works very well for use in cytological evaluation and FISH analysis. How these ethanol-based fixatives work for preserving molecular targets and the ability to isolate analytes properly for use in other molecular methods has not been evaluated in detail. Several urine protein markers, e.g. BTSA, Immunocyt™, and NMP22 BladderChek have made it into patient care [21]. In contrast, while there have been many potential molecular urine markers described in the literature including mutations, methylation, and mRNA, none have so far made it into clinical use [22–28].

The feasibility of using molecular urine markers has been shown in prostate cancer where the mRNA urine marker PCA3 has been successfully implemented as diagnostic tool to make repeat biopsy decisions in patients that are at risk for prostate cancer. The test is based on collecting prostate cells that exfoliate from the prostate after a digital rectal exam (DRE) is performed on the patient to release prostate cells into the urinary tract. Nucleic acids, including mRNA, are stabilized by mixing the urine with a proprietary preservative that enables short-term storage and transport at room temperature. For bladder cancer, similar tests have been used in the research setting although currently none of these have made it into patient care.

Patient monitoring after bladder cancer treatment includes cystoscopy and cytology every 3–6 months for at least the first 2 years. Because cystoscopy is an invasive and expensive procedure, there is a constant need to find less invasive and more economically feasible monitoring methods of patients for recurrence of bladder cancer. Molecular urine diagnostics have the potential to aid in both the initial bladder cancer diagnosis and monitoring of patients that have been treated for bladder cancer. There are several commercial preservatives that can stabilize nucleic acids in urine. Using EDTA as preservative has been shown to be a good choice to collect specimens for biobanking and research. After collection, the urine needs to be processed as soon as possible preferably within two hours. The specimen can be cooled but care should be taken not to over-cool to prevent urea precipitation that will be

difficult to re-dissolve. For quality control purposes it is recommended that urinalysis be performed on all urine specimens that are collected to identify hematuria or infection. The remaining urine after urinalysis can be mixed with EDTA preservative (0.5 mM EDTA 30:1 v/v) that will stabilize the specimen and allow for short-term storage and shipment if needed. The sample is centrifuged to pellet the exfoliated bladder cells. After removal of the urine supernatant, the pellet is resuspended in PBS and either frozen directly as suspension or centrifuged and frozen as a cell pellet. Pellets can be used for nucleic acid isolation and molecular analysis. Recently there have also been efforts to use urine sediments and supernatants as a source for developing urine tumor DNA (utDNA) assays, as a variant of the cell-free circulating tumor DNA assays (liquid biopsy), based on analyzing the utDNA for mutations and or methylation patterns shown to be associated with bladder tumors. While promising, these assays have not yet made it into the clinic. That is also the case with the liquid biopsy assays attempting to use cell-free circulating tumor DNA (ctDNA) and/or circulating tumor cells in blood that have not held up to promise in tumors from any site. The current liquid biopsy assays in development for bladder cancer, although promising, are not ready for clinical implementation [29–33].

Conclusions

With the increasing growth and demand for additional molecular testing to further characterize urothelial malignancy and aid in a more personalized patient management there will be increasing expectations on the pathologists. With increasing demand on pathological tissue both for ancillary diagnostics and research purposes, the proper prioritization has to be made, and the pathologist increasingly functions as the gateway keeper between the clinician managing patient care and the molecular laboratory performing the testing. The pathologist is placed in the position of determining whether and which material will be made available for molecular diagnostics. To accurately determine adequacy of specific tissue for molecular diagnostic this will require knowledge of the molecular methods that are used in testing. As described above, in many cases available surgical material from cystectomy might be limited, not available, or effected by treatment. This will require testing to be done on smaller tissue specimens such as biopsy or TURBT specimen or developing improved methods of testing minimally-invasive techniques such as voided urines or barbotage specimens. Ultimately, it requires a change in mindset on the way routine surgical specimen are handled, processed, and evaluated. As the era of "personalized medicine" evolves, it will become increasingly important that, in addition to routine pathological evaluation, specimens be handled in a manner that will allow for beneficial ancillary molecular diagnostics, and that this should be taken into consideration throughout the whole process of procurement, processing, evaluation, and storage.

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Chapter 7
Diagnostic, Prognostic, and Predictive Biomarkers on Bladder Tissue and Blood

Ryan Hutchinson and Yair Lotan

Introduction

The current understanding of the pathogenesis of bladder urothelial carcinoma (UCa) involves two distinct pathways [1]. One pathway results in non-invasive papillary tumors and is associated with gain-of-function mutations in oncogenes such as fibroblast growth factor receptor (*FGFR3*), *HRAS,* and less often phosphoinositide 3-kinase (*PI3K*). The second pathway results in high grade and frequently invasive tumors and is associated with inactivation and loss-of-function mutations of tumor suppressor genes, e.g. *TP53*, phosphatase and tensin homologue (*PTEN*) and retinoblastoma (*RB1*) [2]. Further understanding of invasive tumors came from the Cancer Genome Atlas analysis, which comprehensively characterized 131 invasive UCa specimens and found statistically significant recurrent mutations in 32 genes, including multiple genes involved in cell-cycle regulation, chromatin regulation, and kinase signaling pathways [3]. While clinicopathological variables have long been recognized as prognostic factors in patients with UCa, more recent insights into the molecular pathogenesis of UCa have augmented this knowledge with additional biomarkers derived from the tumor tissue itself [4, 5]. In UCa, tumors of a similar stage and grade can behave in divergent fashion. There is variability in likelihood of metastasis, as well as response to therapy. Even the best available prognostic models fail to accurately predict outcome more than 20% of the time [6, 7].

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The hope in tissue and blood-based biomarkers research is that markers that are based on molecular pathway alterations may differentiate the cancers predisposed to aggressive behavior and metastases. By extension, tumor-tissue based markers could also identify patients who would be more likely to respond to treatment, whether non-specific (as with cytotoxic chemotherapies) or targeted (as with newer gene inhibitor molecules). Markers may also be used in identifying those patients who are unlikely to require multi-modal therapy, thereby sparing some patients the added toxicity of these more intensive regimens. While markers can correlate with known clinicopathological factors such as tumor grade and stage, simple associations with known clinical data do not add value. Markers, then, seek to achieve independent predictor status, adding diagnostic and prognostic information that would not otherwise be available.

The main factors that impact the utility of cancer-related biomarkers are the differential expression of these markers in tumors as compared to normal cells and the differential expression of markers in tumors that behave in a particular fashion. For example, if a biomarker (protein, DNA, RNA, or genetic modifiers) correlates with tumor behavior beyond that predicted by clinical information alone, the biomarker can add value to clinical management. While many biomarkers have been described, it is important to note that several steps must occur between discovery and correlation to clinical application. The full scope of the discovery and validation of biomarkers is beyond this chapter, but steps in the process are typically followed in an orderly fashion: initial preclinical bench research to identify biomarkers and their mechanisms, pilot testing of an assay in a small known cohort, internal validation on large single-institution tissue arrays and subsequent external validation with multi-institutional samples. Despite decades of near-exponential growth of articles in the arena, few have completed this validation course and no blood or tissue based biomarkers are currently standard of care in UCa [8]. Current cutting-edge efforts are focused on the clinical translation of multiplexed marker panels to answer specific questions in the management of UCa; a table of efforts to identify and verify biomarkers in UCa is included in Table 7.1. In this chapter, we will focus on tissue and blood-based biomarkers, with urine biomarkers addressed in the following chapter.

Cell-Cycle Related Genes

p53, pRB, p21, and p27

Many initial targets for biomarker research were the classic oncogenes such as *RB1* and *TP53*. Cell cycle regulators are among the most commonly mutated markers in bladder cancer and have been shown to be independent predictors of aggressiveness, metastases, and cancer-specific mortality in multiple retrospective and prospective studies [3, 9–13]. While p53 plays an important role, there are similar and/or downstream

Table 7.1 Overview of blood and tissue based biomarker

Overview of biomarkers in urothelial carcinoma	Marker	Type of biomarker	Normal function of gene	Representative studies in urothelial carcinoma
Tissue-based biomarkers				
Cell-cycle based markers	p53	Protein (IHC)	Cell cycle progression inhibitor	Shariat et al. [89]
	pRb	Protein (IHC)	Cell cycle regulator, inhibits E2F transcription factor	Shariat et al. [90]
	Ki67	Protein (IHC)	Marker for interphase of cell cycle	Shariat et al. [25]
	Cyclins, p21, p27	Protein (IHC)	Cyclin-dependent kinases regulate G1 and S phase progression	Wang et al. [91]
Apoptosis and DNA repair based markers	Caspases	Protein (IHC)	Pro-apoptotic proteases	Karam et al. [39]
	Survivin	Protein and mRNA	Anti-apoptotic caspase inhibitor	Shariat et al. [36]
	Fas/FasL	Protein (IHC)	Pro-apoptotic transmembrane signaling complex	Yamana et al. [92]
	MRE11/MRN complex	Protein (IHC)	Repairs double stranded DNA breaks	Choudhury et al. [44]
	ERCC1	Protein (IHC)	Part of damaged base pair excision machinery	Choueiri et al. [41]
Transmembrane and signaling based markers	MDR-1	mRNA expression	Transmembrane efflux pump for toxic compounds including chemotherapy	Hoffmann et al. [51]
	BRCA	mRNA expression	DNA damage repair mediation	Font et al. [46]
	ErbB	Protein (IHC)	Receptor tyrosine kinase involved in cell cycle regulation	Gunes et al. [93]
	PI3K	Protein (IHC)	Signal transduction kinases involved in MTOR pathway and cell cycle regulation	Korkolopoulou et al. [94]
	FGFR3	Protein (IHC)	Growth factor receptor	Van Rhijn et al. [95]
	HER2	Protein (IHC)	RTK for human epidermal growth factor	Schneider et al. [53]
	TSC1	Protein (IHC)	Regulation of MTOR signaling	Iyer et al. [55]
	PD/PDL1	Protein (IHC)	Cell-T cell signaling of normalcy	Kim et al. [56]

(continued)

Table 7.1 (continued)

Overview of biomarkers in urothelial carcinoma	Marker	Type of biomarker	Normal function of gene	Representative studies in urothelial carcinoma
Extracellular markers	Microvessel density	Histology	Measure of tumor and locoregional vessel density	Bochner et al. [96]
	VEGF	Protein (IHC)	Vascular growth factor	Miyata et al. [97]
	Cadherins	mRNA expression	Intercellular connective structures	Muramaki et al. [65]
	Cyclooxygenases	Protein (IHC)	Prostaglandin and thromboxane synthesis enzyme	Aziz et al. [61]
Multiplexed markers	20-Gene panel	mRNA expression	Algorithm-derived panel of genes most predictive of node positivity at cystectomy	Smith et al. [67]
	CoXen	mRNA expression	Algorithm correlating mRNA expression with drug efficacy	NCT02177695, Smith et al. [98]
	Genome DX	mRNA expression	Proprietary mRNA panel	Lotan et al. (in progress)
	NCI-match	Sequencing	Sequencing-derived treatment recommendations in second line for multiple malignancies	Flaherty et al. (in progress)
Blood-based biomarkers				
General blood-based biomarkers	Circulating tumor cells	Tumor cells isolated from blood	Finding in multiple solid organ tumors generally associated with more advanced disease	Rink et al. [75]
	CD8 Count	Circulating cytotoxic T cell levels	Immune surveillance of tumors, killing of abnormal cells	Lin et al. [76]
	C-reactive protein	Acute phase protein	Activates compliment system	Masson et al. [79]
	IGFBP-3	Binding protein for insulin growth factor	Transmits signal of insulin growth factor	Shariat et al. [83]
	TGF-β	Circulating hormone	Anti-proliferative and pro-apoptotic signaling	Shariat et al. [81]
	Matrix metalloproteinase	Proteinase targeting extracellular matrix	Remodeling of extracellular matrix	Svatek et al. [84]

(continued)

pathways that contribute to urothelial transformation which can modify the effects of p53 [13–15]. For example, members of the kinase inhibitor protein family, $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, are p53-inducible and p53-independent cyclin dependent kinase inhibitors that can arrest the cell by inhibiting DNA replication [9, 10, 12, 13, 15–21].

In 2010 Shariat et al. examined four classic cell-cycle regulators, p53, pRB, p21, and p27, in a retrospective cohort of 700 patients with locally advanced disease and found that a combination of the four markers was able to significantly, but modestly, improve prognostication for recurrence and cancer-specific mortality [22]. While this independent predictor status remained for pT3/4 and N+ subgroup analyses, the improvements over a standard clinicopathological prognostic model remained small. Perhaps because of the redundancy of pathways or the inability of p53 to predict response to chemotherapy, a trial from the Southwest Oncology Group (SWOG) utilizing p53 to randomize patients to adjuvant chemotherapy did not show a benefit [23]. There are prospective studies that did demonstrate that a panel of markers including p53, p21, p27, cyclin E, and Ki67 did improve prediction of recurrence after cystectomy over clinical factors alone [24] and may improve prediction of upstaging after transurethral resection (TUR) [25]. At this time, the most robust information regarding predictive markers involves cell cycle regulators such as p53 but there are intrinsic issues related to immunohistochemistry including interpretation and many of the studies thus far have been retrospective. Clinical application is still limited.

Cyclins

Cyclins are cell cycle regulatory proteins that have been examined as proxies for tumor aggression across a wide range of tumor types. In UCa, cyclin D1 and E1 immunostaining was examined in a tissue microarray of patients with non-invasive UCa (Tis, Ta, and T1) and were found to be predictive of tumor recurrence and progression in univariate, but not multivariate analysis [26]. Similar findings were observed when immunohistochemistry for a panel of cell cycle proteins including p16, p21, p53, and cyclin D1 was performed, calling into question the utility of this gene product as a prognostic test [27]. Subsequent analysis of subtypes of cyclin D revealed cyclin D1 to be more important in early stage tumors while cyclin D3 expression appeared to be associated with a more aggressive phenotype in muscle invasive disease and did achieve independent predictor status in a 2010 immunostaining study of 150 patients [28].

Ki-67

Cell proliferation is a hallmark of cancer. Ki-67 is a nuclear protein expressed by proliferating cells and is a measure of cell growth fraction. Cell proliferative index is associated with prognosis in non-muscle invasive UC and Ki-67 antigen is an

independent predictor of recurrence, progression, and response to immunotherapy in patients with non-muscle invasive UCa [29, 30]. This finding has been externally validated in patients with muscle invasive bladder cancers undergoing cystectomy [31, 32].

Apoptosis-Mediating Genes

Apoptosis is the process of programmed cell death that results from a combination of intrinsic and extrinsic signals that converge into a common downstream effector pathway [33]. Abnormalities in apoptosis are important factors in carcinogenesis because they facilitate survival of cancer cells, improve the ability of cells to develop resistance to harmful stresses, and increase invasiveness with resultant progression and metastasis. The apoptotic cascade involves multiple proteins with the resultant activation of caspase-3, a proteolytic effector molecule that acts downstream in the apoptosis pathway, resulting in cellular disassembly. Genes such as *TP53* (discussed above) function in induction of cell-cycle arrest and apoptosis. Conversely, Bcl-2 is an anti-apoptotic protein that controls ion channels, caspase status, and cytochrome c location [34]. Survivin is also an inhibitor of apoptosis which can bind caspases after their activation, therefore preventing them from cleaving their substrates [35]. Survivin expression has been shown to be associated with bladder cancer progression and mortality and its function as a prognosticator has been externally validated [36–38]. In a multiplexed panel including other apoptosis-regulating genes, it has been shown to predict post-cystectomy recurrence and mortality more accurately than clinicopathological factors alone [39].

DNA Repair Genes

ERCC1

The excision repair cross-complementing 1 (*ERCC1*) gene codes for one protein subunit in a DNA repair complex responsible for multiple critical portions of DNA repair, especially the excision of damaged or crosslinked nucleotides. Since platinum-based chemotherapy acts by disrupting DNA, the ability to repair this damage by tumors may impact chemosensitivity. To that end, tumor alterations in these genes have been examined as markers to predict response to chemotherapy-containing bladder cancer treatment algorithms. A study of immunohistochemical staining for DNA excision repair mutations demonstrated independent predictor status with respect to disease-specific survival in patients with bladder cancer treated with tri-modality (maximal transurethral resection, systemic chemotherapy, and local radiation) therapy [40].

The prognostic ability of ERCC1 has subsequently been examined in two other clinical scenarios: post-cystectomy to assess risk of recurrence and at the

time of neoadjuvant chemotherapy to examine pre-surgical responses to methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC). In 2014 Choueiri et al. examined the safety of a dose-dense MVAC regimen with pegfilgrastim in the neoadjuvant setting and found a favorable safety profile but no association between ERCC1 expression and tumor response or survival [41]. A 2015 study of patients undergoing radical cystectomy also found no association between ERCC1 expression and response to neoadjuvant chemotherapy but did find that ERCC1 expression was a significant independent predictor of clinical endpoints with ERCC1 expressing tumors having significantly longer disease-free survival (HR 0.70, $p = 0.028$) and cancer-specific survival (HR 0.70, $p = 0.032$) [42].

MRE11 and MRN Complex

MRE11 is a protein product that plays a role in repairing double stranded DNA breaks as part of the MRN complex. In muscle invasive bladder cancer a triple therapy regimen including maximal transurethral resection of tumor, systemic chemotherapy and radiation therapy to the bladder has been proposed and appears associated with acceptable short and intermediate term oncologic outcomes [43]. MRE11 expression levels have been shown to be a predictive factor when assessing disease-specific survival in patients treated with triple therapy for muscle invasive disease [44], which may have value in pre-therapy prognostication for these patients. Another study found that carriage of at least one of six rare variants of the MRE11A gene was associated with the worse RT outcome (hazard ratio [HR] 4.04, 95% confidence interval [95% CI] 1.42–11.51, $P = 0.009$) [45]. If validated, markers may help patients decide whether to undergo chemoradiation regimens based on likelihood to respond to this therapy.

BRCA1 Expression

BRCA1 is a well-known oncogene implicated in familial cancers including breast and prostate. It is involved in DNA repair and so has been examined as a marker for susceptibility to DNA-targeting chemotherapeutics including cisplatin. In the neoadjuvant setting, BRCA1 mRNA expression levels have been shown to be prognostic in patients receiving cisplatin-based chemotherapy regimens before cystectomy with 66% of patients with low BRCA1 expression achieving pT1–0 status versus 22% with high BRCA1 expression [46]. In the metastatic setting a 2015 study examined BRCA1 protein expression on a tissue micro array with immunohistochemical staining. While other DNA repair proteins were shown to be predictive of response to cisplatin-based chemotherapy, BRCA1 was not [47].

Cathepsin E, PLK1 and Serpin B5

Cathepsins are ubiquitous proteases often active within cellular lysosomes. The protein expression of cathepsins in urothelial carcinomas has been studied for over 10 years as a possible proxy for the ability to invade and metastasize [48]. In a 2012 study Danish researchers examined Cathepsin E, maspin, Plk-1, and survivin as protein biomarkers for progression in non-muscle invasive (Ta and T1) urothelial carcinoma [49]. In a cohort of 690 patients, they found immunohistochemical staining of cathepsin E, Plk-1, maspin and survivin to be significant independent prognosticators (HRs 0.31, 1.68, 0.44, and 2.25, respectively) for disease progression to muscle invasive (T2) bladder cancer. A composite signature of the four markers was composed with significant prognostic ability (HR of high composite score: 6.48) for progression which could have clinical utility in differentiating patients for aggressive transurethral management versus early cystectomy.

Transmembrane and Signaling Genes

mTOR Gene Family

The mammalian target of rapamycin (mTOR) pathway is dysregulated in multiple solid organ malignancies and medications targeting this pathway (mTOR inhibitors) are a staple of metastatic disease management in renal cell carcinomas. In UC this class of medications is not routinely used, likely owing to the fact that the mTOR pathway is generally downregulated in muscle invasive bladder cancer (MIBC) [50]. When individual pathway genes (PTEN, c-myc, p27, phosAkt, phosS6) were assessed for prognostic status in a model including TNM staging and histology, phosS6 expression was an independent predictor of disease-specific survival and c-myc was an independent predictor of progression [50].

MDR1

MDR1 is a transmembrane protein responsible for the efflux of toxic intracellular compounds. It has been implicated as a primary driver of the high degree of chemoresistance in renal cell carcinomas. MDR1 exhibits variable expression in urothelial carcinomas. In 2010 Hoffman et al. used quantitative PCR on tissue from a prospective trial for locally advanced bladder cancer and found that high levels of MDR1 expression were associated with inferior clinical responses to adjuvant platinum-based chemotherapy [51]. Similar results were seen with the excision repair cross complimenting gene (ERCC1), with a slightly lower relative risk for progression free survival (2.24 vs 2.9) [51]. Similar findings were observed when ERCC1 mRNA levels were used to stratify metastatic urothelial

carcinomas with those tumors with high expression having significantly shorter median survival (15.4 vs. 25.4 months) in patients treated with DNA-targeted chemotherapy regimens [52].

HER2

HER2 is an extracellular receptor tyrosine kinase for human epidermal growth factor. In UCa, HER2 mutations have been noted frequently in patients with variant histology urothelial carcinomas, specifically in plasmacytoid and micropapillary variants which carry a worse prognosis than standard urothelial carcinomas. Even within the subgroup of poor-risk bladder cancer patients with micropapillary tumors, those with HER2 amplification have been shown to have a worse prognosis than those without [53, 54].

TSC1

TSC1 is a tumor suppressor gene and a portion of a larger complex which regulated mTOR signaling. It has been implicated as a tumor suppressor gene and TSC1 mutations are a causative agent in tuberous sclerosis. TSC1 has been examined as a prognostic biomarker in Ta and T1 urothelial carcinomas; using TSC1 either alone or as a part of a three biomarker panel did not add predictive value for the assessment of recurrence or progression. Conversely, a single-patient report using whole genome sequencing to evaluate a dramatic response of metastatic urothelial carcinoma to everolimus discovered a somatic mutation in *TSC1* in the index patient and subsequently demonstrated an 8% mutation rate in a panel of urothelial carcinomas, which correlated with treatment response to everolimus [55].

PDL/PDL1

The programmed-death ligand system is an area of recent focus in the study of metastatic urothelial carcinoma. The transmembrane protein pair allows normal cells to interact with CD8 (cytotoxic) T cells to inhibit their local proliferation and activation. A large number of studies are currently in progress with targeted agents blocking this interaction [56] and unique associations have arisen from examining the PD/PDL axis in urothelial carcinoma. Aberrant expression of PDL1 is associated with advanced grade and stage in UCa [57]. Interestingly, PDL expression on tumor-infiltrating monocytes was associated with favorable survival outcomes in patients treated for metastatic UCa while PDL expression of tumor cells themselves was not [58]. A similar lack of association was found in a subsequent study where tumor-infiltrating CD8 cell density was associated with favorable overall and disease-specific survival outcomes, while PDL expression on tumor cells was not [59].

Extracellular Environment Related Markers

COX-2 Expression

COX-2 is an enzyme most commonly known as a target for non-steroidal anti-inflammatory drugs such as ibuprofen and celecoxib. COX-2 overexpression has also been examined as a marker of UCa angiogenesis and tumor aggressiveness in both the upper and lower tracts. In 2003 Friedrich et al. [60] reported on 110 patients with non-muscle invasive bladder tumors (NMIBC) and found that COX-2 immunostaining was predictive of tumor microvessel density but not tumor recurrence [60]. In MIBC, COX-2 staining has been proposed as a metric to measure the body's immune response to an invasive tumor with one study showing better disease outcomes in COX-2 positive patients with T2 or greater tumors undergoing cystectomy [61]. In upper tract tumors similar findings have been observed with COX-2 staining of tumor tissue, however increased COX-2 staining of adjacent (non-tumor) stromal cells has been shown to be associated with more aggressive upper tract tumors and worse clinical outcomes [62–64].

Cadherin mRNA Profiles

Cadherins are calcium-dependent transmembrane proteins that mediate cell–cell binding. Loss of cadherin expression has been implicated as a primary marker of the epidermal to mesenchymal transition that occurs in many malignancies as they de-differentiate, become more aggressive and metastasize. Cadherin type switching has been examined as a potential molecular marker in UCa. In a 2012 study N-cadherin-expressing non-invasive UCa had significantly higher rates of intravesical recurrence (69 versus 11%) when compared to E-cadherin expressing tumors [65]. E-cadherin expression loss has been shown to be a significant negative prognosticator for progression and disease-specific survival in patients with CIS of the bladder as well [66].

Multiplexed Marker Panels

Twenty-Gene Model for Urothelial Carcinoma

Multiplexing of gene assays has the potential to further improve prognostic ability, especially for specific, clinically relevant decision points. Examples include prediction to inform which patients would benefit most from neoadjuvant chemotherapy and prediction as to which patients with clinically localized disease are likely to

harbor micrometastases. The first multiplexed gene model for UCa validated in a prospective cohort was published in 2011 and comprised a screen of potential gene targets in an initial cohort to arrive at a 20-gene panel that was predictive of nodal metastases in patients with clinically localized disease [67]. This panel was then validated using tissue from a previously conducted prospective trial comparing different adjuvant chemotherapy regimens in patients with locally advanced disease. While the panel performed well in both cohorts, the relative risks for high versus low scores were modest (1.74 and 0.70) and there was no identifiable cut point which would result in a test with either very high negative predictive value or very high positive predictive value, which would be clinically preferable.

GenomeDX Gene-Expression Assay

GenomeDX is a company offering a commercially available 22 Gene assay for the prognosis of men with prostate cancer who undergo prostatectomy [68]. The applicability of this multiple-gene assay to assist in treatment decisions in other urologic malignancies is ongoing. In urothelial carcinoma specifically the decision to administer neoadjuvant chemotherapy prior to cystectomy is supported by level one evidence, however penetration of this practice is limited [69]. Additionally, patients with more advanced disease tend to benefit more from neoadjuvant chemotherapy leading to concerns for overtreatment [70, 71]. The GenomeDX molecular assay is currently being evaluated in a cohort of patients who underwent cystectomy to assess for genomic predictors of advanced tumor stage at cystectomy using the assay on tumor tissue obtained from transurethral resection specimens (study in progress). If improved prognostication can further aid in differentiating which patients will best benefit from chemotherapy before cystectomy, the practice may become more widespread.

Biomarkers for Marker Phenotype Targeted Therapies

While many malignancies have recently been more effectively treated with gene-targeted agents rather than cytotoxic chemotherapy, the use of targeted agents in urothelial carcinoma has not yet produced new first-line regimens. A criticism of many trials of targeted agents in UC has been a lack of pre-treatment tumor sequencing, especially in a malignancy as genetically heterogeneous as UC. Basket studies have emerged as a possible solution where, instead of comparing one treatment to another, treatment pathways involving pre-therapy sequence-directed care are compared to standard of care [72]. This study design may become the standard in treatment of non-localized disease as the armamentarium of targeted therapies expands.

COXEN Trial (S1314)

Currently, two chemotherapeutic regimens (MVAC and gemcitabine plus cisplatin) are commonly used in the neoadjuvant setting for patients undergoing cystectomy. The ability to predict response to chemotherapy is important since patients with low likelihood of response might be able to either avoid unnecessary therapy or enroll in other experimental protocols. The COXEN principle (coexpression extrapolation) uses expression microarray data as a guide for translating from drug activities derived from an early sample of 60 malignancies (NCI-60) to drug activities in any other cell panel or set of clinical tumors. This approach was shown to have promise in a 2007 study which screened 100,000 compounds against the NCI-60 and found that treatment response could be predicted from expression profiles in urothelial and breast cancers [73].

One trial that is underway (S1314, COXEN) seeks to provide a basis for prospectively validating this principle in vivo (NCT02177695). Patients in this trial will be randomized to either MVAC or GC and have their tumors profiled by a multiplexed marker panel to assess for marker profiles associated with favorable response (clinical absence or regression of tumor at surgery) to these neoadjuvant chemotherapy regimens. The end goal is the development of a marker panel that can be administered to all patients planning to undergo neoadjuvant chemotherapy to allow the choice of regimen to be made not by provider preference, but by optimal probability of a robust response to therapy.

NCI-MATCH Trial

A further iteration of algorithm-derived biomarker panels is the concept of directing systemic therapies against generalized tumor "phenotypes" which may respond particularly well to certain subsets of chemotherapeutic or targeted agents. A trial currently underway (NCT02465060) to test this approach versus standard-of care (which would be standard second-line targeted therapies or chemotherapies for the given tumor origin) is NCI-MATCH, in which patients in the intervention arm with a wide variety of solid organ and blood-based malignancies will undergo tumor sequencing and treatment selection based on molecular marker phenotype [74].

Blood-Based Biomarkers

Multiple serum-based biomarkers have been examined for both upper and lower tract UCa. These targets have run the gamut from circulating tumor cells to markers of systemic inflammatory state. At current no blood-based markers for urothelial carcinoma are routinely in clinical use.

Circulating Tumor Cells

The simplest blood-based biomarker is that of circulating tumor cells. In multiple solid organ malignancies, the presence of tumor cells in the blood has been associated with advanced stage disease. In UCa, Rink et al. examined the prognostic ability of circulating tumor cell counts captured by the CellSearch system in 100 patients undergoing cystectomy [75]. They found one quarter of clinically localized UCa patients had detectable circulating tumor cells and, after correction for clinicopathological variables, found significantly increased risk for recurrence, cancer-specific mortality, and overall survival with hazard ratios of 4.6, 5.2, and 3.5, respectively.

Circulating Immune Cell Differentiation

CD8 count is a measure of the presence of cytotoxic T cells which may be involved in tumor immune surveillance. CD8 count in peripheral blood was assessed in one study by flow cytometry and found to be inversely correlated with tumor CD8 cell infiltration; this study also noted that lower levels of peripheral CD8 cells were associated with greater freedom from recurrence in patients treated with transurethral resection of bladder tumor [76]. A proxy for acute phase inflammation, the neutrophil-to-lymphocyte ratio, has also been examined as a measure of tumor aggressiveness. At least two studies have confirmed that a neutrophil-to-lymphocyte ratio of greater than 2.5 was independently predictive of cancer-specific mortality [77, 78].

C-Reactive Protein

Additional blood-based markers have ranged from systemic proxies of pro or anti-inflammatory state to extracellular proteins not normally present in blood. One of the most studied markers has been C-reactive protein, a marker for inflammation. A 2014 meta-analysis summarized these findings and identified eight studies with the consistent finding that elevated blood levels of C-reactive protein were associated with adverse oncologic outcomes [79]. Similar findings of worsened recurrence free survival and disease-specific survival in patients with elevated C-reactive protein have been demonstrated in upper-tract only studies as well [80].

TGF-β1

Transforming growth factor β1 (TGF-β1) is a multiple-pathway signaling factor that has been implicated in de-differentiation of tumor cells. Shariat et al. examined the effect of pre-surgical levels of TGF-β1 in a cohort of 50 patients undergoing

cystectomy and compared to a similar number of healthy controls. The authors found that levels of TGF-β1 were greater in patients with bladder cancer and levels of TGF-β1 exhibited a dose-response with higher levels found in patients with greater T stage and nodal metastases [81]. The receptor of TGF-β1 has multiple common small nucleotide polymorphisms present throughout the population at large. In 2009 these were studied for predictive power in a sample of 1200 patients with urothelial carcinoma and matched with a cohort of healthy patients. While the SNPs were not predictive of outcomes in non-invasive disease, a single SNP (rs868) was associated with disease-specific mortality in patients with muscle invasive disease [82].

IGF and IGFBP-3

Insulin growth factor (IGF) and insulin growth factor binding protein (IGFBP-3) are circulating proteins that act as growth signaling intermediaries and mitogens. Their role as blood-based predictors for outcomes in UCa was examined in a 2003 study of 50 patients undergoing cystectomy who had preoperative IGF and IGFBP-3 levels drawn. While individual assessment of marker levels was not independently predictive of outcomes, a combined marker of low IGF-adjusted IGFBP-3 level was predictive of nodal metastases and poorer survival outcomes [83].

Matrix Metalloproteinase

Matrix metalloproteinase (MMP) is a zinc-dependent endopeptidase responsible for lysis of multiple extracellular matrix proteins. Circulating levels of various MMP isoforms were evaluated in a cohort of invasive (T1 or greater) bladder cancer patients and MMP-7 levels specifically were associated with cancer-specific mortality after adjustment for clinicopathological variables [84]. Subsequent work by Szarvas et al. evaluated serum and tissue levels of MMP-7 across a range of clinical stages and found elevated serum MMP-7 levels in the serum of patients with metastatic disease as well as independent prognostic value for metastasis-free survival and cancer-specific survival [85].

Periplakin

Periplakin is a protein encoded by the PPL gene that is a portion of normal cellular desmosomes. Serum circulating periplakin has been examined in a cohort of Japanese UCa patients and compared to 30 normal controls. While serum periplakin levels were significantly lower in UC patients versus controls, this difference narrowed in patients with muscle invasive disease [86].

Epigenetic Tumor Markers

Epigenetics is a newer branch of genetic analysis that looks at molecular DNA changes including methylation and histone alterations including acetylation instead of classic base pair coding. While the genetic code contained within DNA is generally unchanged within an organism, DNA methylation and histone acetylation are dynamic processes that can change what parts of DNA are more frequently transcribed and which regions are silenced. For this reason epigenetic changes present in urothelial carcinomas have been examined as potential biomarkers for tumor behavior.

A British study compared promoter methylation of 17 genes associated with tumor progression in 96 urothelial carcinoma samples compared to 30 samples of normal urothelial tissue. Promoter methylation of five gene loci (RASSF1, E-cadherin, TNFSR25, EDNRB and APC) were identified as predictors for tumor progression [87]. A larger study, also from England, examined 280 patients with both upper and lower tract urothelial carcinomas. This study found a higher proportion of upper tract tumors exhibited targeted promoter methylation (94 vs 76%) than bladder tumors and identified methylation at the RASSF1A and DAPK loci as independent predictors for tumor progression [88].

Conclusion

As the understanding of tumor biology deepens, a greater array of diagnostic and prognostic tools are becoming available to urologists. In urothelial cancer, there are a myriad of gene products as targets for evaluating tumor behavior and providing targeted therapies. In the coming years a clearer picture will emerge as to which have clinical value and how this additional information can translate into better outcomes for patients.

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Chapter 8
Urine Cytology and Existing Urinary Biomarkers for Bladder Cancer

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Urine-based testing would seem to be the obvious diagnostic choice for bladder cancer. Conceptually, an ideal diagnostic test would be simple and application of the test would determine if the disease is present or absent. However, like all diagnostic tests for cancer, urine-based tests for bladder cancer suffer from poor performance, limited clinical utility, and the potential for introducing harm. Consequently, none are universally recommended diagnostic tests for use in the evaluation of patients at risk of having bladder cancer [1–3]. Despite this fact, extensive investment into the research and development of urine-based technologies promising to be better bladder cancer tests continues to be made [4].

Test Performance Characteristics for Urine-Based Tests

Diagnostic test accuracy can be summarized using the QUADRAS-2 tool [5], and the STARD initiative was developed to make reporting of diagnostic accuracy studies complete and transparent [6]. Describing the performance of a urinary test is usually done using several metrics including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), the diagnostic likelihood ratio positive (DLR+), and the diagnostic likelihood ratio negative (DLR-) [7]. It is important to recognize that the calculation of these parameters requires that the test result be binary; either positive or negative. For non-binary tests with results reported on ordinal or continuous scales, alternative methods are available (e.g., receiver operating characteristic (ROC) curves) that summarize test

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Table 8.1 Diagnostic contingency table

		Disease	
		Present	Absent
Test	Positive	a	b
	Negative	c	d

performance appropriately. In these cases of non-binary results, however, "optimal" thresholds are often selected by researchers in order to dichotomize the test. This allows results to appear binary and therefore make more straightforward binary result analysis possible. However, it is important to recognize that dichotomizing a continuous test using a sample-driven threshold can lead to several biases and should be undertaken with great care [8, 9].

Using Table 8.1, the *prevalence* of disease in the sample is calculated as $\rho = \frac{a+c}{a+b+c+d}$. *Sensitivity* is the probability of a positive test if the subject truly has the disease and is calculated by $Sens = \frac{a}{a+c}$. *Specificity* is the probability of a negative test if the subject truly is disease-free and is calculated by $Spec = \frac{d}{b+d}$. Note that since the specificity and sensitivity are calculated within the columns of the contingency table, they are not affected by the prevalence of disease in the sample. This means that the sensitivity and specificity of a test is independent of how rare the disease of interest is in the sample population. Furthermore, these parameters should be similar in cohort and case-control designs which utilize different sampling methods. While sensitivity and specificity are not affected by disease prevalence, they are affected by disease severity, something known as *spectrum bias* [10–12]. Generally when the disease severity/burden is high, the test sensitivity will appear better and the specificity will appear lower.

The *positive predictive value* is the probability of having the disease if the test is positive and is given by $PPV = \frac{a}{a+b}$. The *negative predictive value* is the probability of not having the disease if the test is negative and is given by $NPV = \frac{d}{c+d}$. Unlike sensitivity and specificity, the predictive values are calculated from the rows of the contingency table and are therefore directly affected by the prevalence of disease in the sample population. Predictive values will vary when the same test is applied to different patient subgroups that have different risks of disease. For example, *referral bias* occurs when a diagnostic test is applied to a sample taken from a specialty clinic with a higher than expected disease prevalence.

Predictive values must in turn be distinguished from diagnostic likelihood ratios. The *positive diagnostic likelihood ratio* is given by $DLR+ = \frac{Sens}{1-Spec}$ and the

Table 8.2 Interpretation of diagnostic likelihood ratios

Value	DLR+	DLR−
0	Positive test perfectly rules out disease	Negative test perfectly rules out disease
0–1	Positive test decreases probability of disease	Negative test decreases probability of disease
1	Uninformative test	
1–∞	Positive test increases probability of disease	Negative test increases probability of disease
∞	Positive test perfectly rules in disease	Negative test perfectly rules in disease

negative diagnostic likelihood ratio is given by $DLR- = \frac{1 - Sens}{Spec}$. Like sensitivity and specificity, the diagnostic likelihood ratios are calculated along the columns of the diagnostic contingency table and are therefore independent of disease prevalence. The diagnostic likelihood ratios quantify the increase in knowledge about the presence/absence of disease that is gained by applying the test, something that becomes very important in Bayesian decision-making frameworks. The interpretation of the diagnostic likelihood ratio is given in Table 8.2.

The Gold Standard for Bladder Cancer Diagnosis: Cystoscopy

The gold standard against which most diagnostic tests for bladder cancer are measured is white light cystoscopy (WLC) [3, 13]. While WLC is considered extremely sensitive and very specific for bladder cancer, it remains costly and somewhat invasive. Newer augmented cystoscopy methods such as hexaminolevulinate photodynamic diagnosis (PDD) and narrow-band imaging (NBI) [14–16] have been developed with the goal of increased sensitivity, however they are even more costly and have not yet become the community standard [1, 17–19]. Tumor histology obtained from cystoscopic biopsy specimens is an inappropriate gold standard for the evaluation urine test for two reasons. First, histology results are available only after the decision to biopsy has been made. This is the key decision point (biopsy or no biopsy) that cystoscopy and urine tests are trying to inform. It is not possible to use results that occur *after* a decision to inform that decision (e.g., you cannot make the decision to bet or fold your poker hand after you know if you won the hand). Second, histology is not available on all patients since only the positive/suspicious cystoscopy and positive urine tests proceed to biopsy. This unequal application of the gold standard to the study population is known as verification bias [20], and it can have very significant impact on how the diagnostic test performance metrics discussed above are interpreted.

Urine test results that are discordant from negative cystoscopy are a significant problem as they may falsely indicate the need for further diagnostic workup. This

risk of this includes morbidity from unneeded extra interventions and testing done to chase down a positive test result (e.g., ureteroscopy and bladder biopsy), the risk of financial consequences of excess testing, the risk of patient worry and anxiety, and the medico-legal risk to the physician of a missed diagnosis. Currently available urine tests for bladder cancer are plagued in the clinic by mediocre performance, a strong dependency on how suspicious/atypical results are handled [21], spectrum bias where the test performs dramatically differently in one group of patients than another [12], cost issues, reader/interpreter dependency [22, 23], an inability to replace cystoscopy [18, 24, 25]. The AUA has recently recommended against using urine cancer tests during microscopic hematuria evaluation for this reason [3], but this argument could be extended to bladder cancer surveillance as well [21].

Anticipatory Positive Tests

Occasionally, a urine-based diagnostic test will be positive while cystoscopy and upper urinary tract evaluation are negative. In these cases, it is possible that either the urine test result is a false positive or that it is, in fact, a true positive which will become clinically apparent after some interval of follow-up when tumors become visible. An anticipatory positive test result refers to these true positives which detect bladder cancer prior to clinical detection by cystoscopy; the gold standard [26–28]. Several criteria can be used to define what constitutes an anticipatory positive test result: (1) the urine test must be positive prior to cystoscopy or upper urinary tract imaging or endoscopy; (2) the probability of developing a positive cystoscopy over time must be higher when the urine test is positive than when it is negative; and both (3) the measured specificity and (4) the sensitivity of the urine test must increase when the cystoscopy results that occur in the future (i.e., the cystoscopy results that show that the prior urine test anticipated the tumor) are credited to the urine test. Some urine tests appear to anticipate future tumors, but do so in such an unpredictable and inconsistent way that this property becomes all but useless. Anticipatory positivity was recently assessed in a large sample of urine cytology and FISH tests and demonstrated that positive urine tests frequently are not anticipating cancer [29].

Spectrum Bias

Sensitivity and specificity (and consequently the diagnostic likelihood ratios) are not fixed test properties and often vary across subgroups. This means that when a urine test is reported to have a particular sensitivity or specificity, this result may not apply to your patient population, a phenomenon that is known as spectrum bias [11, 30–32]. Although reporting the spectrum biases of diagnostic tests is recommended by the STARD initiative, it is an uncommon practice [33]. Sometimes, the differences in test performance can be so dramatic between patient subgroups that the test

becomes very difficult to use. For example, we have shown that urine cytology and Urovysion FISH performance has dramatic variation between patient subgroups [12]. Proper stratification into relevant subgroups during the evaluation of a diagnostic test can highlight important spectrum biases [10].

Combining Diagnostic Test Results

It is common that more than one diagnostic test for a disease is considered. These multiple tests may be obtained sequentially or in parallel. When tests are ordered sequentially, the results of the first test inform the decision to obtain the second, and so on. Sequential testing leads to a decrease in sensitivity and NPV while causing an increase in specificity and PPV. Parallel testing, when a battery of tests is obtained all at once, leads to an increase in sensitivity and NPV while causing a decrease in specificity and PPV. Bayesian methods that use diagnostic likelihood ratios are particularly well suited for the combination of multiple decisions in medical decision making [34].

Hematuria and Bladder Cancer

Hematuria is the presence of microscopic (≥3 RBCs per high-powered microscopy field) or visible blood in the urine [3]. The association of hematuria with the presence of bladder cancer varies greatly in gross versus microscopic hematuria. Bladder cancer has a high prevalence (10–20%) in patients presenting with gross hematuria, indicating a clear need for cystoscopy in this population for detection [35–37]. However, the indication for cystoscopy in patients with microscopic hematuria is far more controversial, as the probability of BC in this setting is only 1–3% [3, 38]. This is complicated further by the high prevalence of microscopic hematuria in the adult population (2–20%) [36, 39], representing millions of adult Americans. Detecting microscopic hematuria is easy and inexpensive; urine dipstick tests have a sensitivity of ~80% and specificity of ~90% [40]. The problem arises when we attempt to use microscopic hematuria evaluation as a screening test for bladder cancer. This is due to the fact that microscopic hematuria itself has a low specificity for bladder cancer [40]. The low prevalence of bladder cancer in the general population therefore has dulled the enthusiasm for generalized microscopic hematuria screening [41–43]. In certain occupational settings where the risk of bladder cancer is felt to be very high, microscopic hematuria screening may make sense, however it is likely inappropriate in the general population. Analysis of a single urine dipstick as a screening tool for bladder cancer for the general population actually results in a PPV of 0.2% and an NPV at 98.8% [44]. Attempts at raising the cutoff for hematuria [45], or performing serial dipsticks have proven only marginally better [46]. Ultimately the low prevalence of BC has rendered broad screening measures ineffective [46].

Urine Cytology

Urine cytology involves looking for exfoliated neoplastic cells in the urine by microscopy and was first described in 1864 [47, 48]. It is the most commonly used urine test in the detection of bladder cancer. The urine cytology procedure involves centrifuging urine to obtain a cellular pellet, washing and resuspending the pellet, smearing the cells on a glass slide, then staining the slide with a Papanicolaou stain (or equivalent). In many centers, a cytotechnologist screens the cells and any abnormal slides go on to second tier evaluation by a cytopathologist (verification bias). Traditionally, urine cytology results are reported as positive, negative, atypical, inconclusive, suspicious, or as an inadequate sample. However, cytology results are not very reproducible and significant intra- and interobserver variation has been observed [49, 50]. Furthermore, urine cytology results are often (25–50%) reported as equivocal (atypical, inconclusive, or suspicious) [12, 51–55], which confounds clinicians and patients [56–58]. Equivocal results have a very large impact on the diagnostic performance of urine cytology and are rarely taken into account in studies of its diagnostic accuracy where test results are assumed to be binary, either positive or negative. When equivocal results are considered, the sensitivity and specificity of cytology worsen dramatically [59]. Adjunctive diagnostic tests have been used to adjudicate equivocal cytologies, as discussed later.

In order to make urine cytology more reproducible, a new classification method called the *Paris system* has been developed [60]. This system is designed to focus on high-grade cytological features (Table 8.3). Surprisingly, the new system includes a review of imaging and cystoscopy reports for certain cytology categories which indicates that diagnostic review bias is a significant possibility [13]. Ideally, the result of the urine cytology test should not depend on the results of other tests. It remains to be seen whether the Paris system will improve cytology performance.

Cytology is generally reported to have a sensitivity of ~30% and a specificity ~95% for bladder cancer, though these overall estimates are likely overly optimistic given more recent findings (see below) [61]. Urine cytology performance also varies significantly between patients. Numerous investigators have found a better sensitivity/specificity for high-grade tumors and worse sensitivity/specificity for low-grade lesions, a manifestation of spectrum bias [56, 57, 62–65]. Low-grade lesions and small tumors are thought to be less likely to exfoliate cancer cells into the urine and consequently are harder to recognize with urine cytology [66]. Other patient factors also affect urine cytology. Increasing age, male gender, and history of smoking are associated with increased sensitivity and decreased specificity [12]. Also, false positive results occur in the settings of instrumentation, inflammation,

Table 8.3 Paris system for reporting urine cytology [60]

1	Non-diagnostic/Unsatisfactory
2	Negative for high-grade urothelial carcinoma
3	Atypical urothelial cells
4	Suspicious for high-grade urothelial carcinoma
5	High-grade urothelial carcinoma
6	Low-grade urothelial carcinoma
7	Other

infection, stones, treatment with chemo and radiotherapy [52]. Despite all these factors affecting urine cytology results and universal acceptance that it has extremely poor sensitivity for bladder cancer, it is still widely used, predominantly because of a prevailing belief that it is rarely falsely positive. Indeed, some positive urine cytology tests have been shown to anticipate some future bladder cancers that are currently invisible with cystoscopy [67]. While this undoubtedly occurs in some cases, other investigators have shown that random bladder biopsies done in normal appearing bladders for positive cytologies has little benefit [68]. In consideration of these limitations, the AUA no longer recommends cytology in the workup of asymptomatic hematuria or in surveillance of low-grade bladder cancer [3].

Several things can be done to improve urine cytology performance. Immediate centrifugation prevents loss of cells due to prolonged processing [66]. Using whole voided specimen and multiple urine samples can increase the sensitivity (though also probably reduces specificity) [69]. Although it is a routine practice to obtain a voided urine specimen, a bladder barbotage obtained at cystoscopy increases the sensitivity for high-grade lesions [70, 71]. However, others have found that instrumentation can be a potential source for a false positive result [72, 73]. Other causes of a false positive cytology include inflammation, infection, stones, treatment with chemo and radiotherapy [52].

UroVysion Fluorescence In Situ Hybridization (Fish)

Fluorescence in situ hybridization (FISH) is the second most commonly used urine test for bladder cancer. UroVysion FISH is a cell-based assay that detects aneuploidy of chromosomes 3, 7, and 17 as well as the deletion of the 9p21 locus in exfoliated urine cells. Though FISH was long known to have the ability to detect bladder cancer [74–76], it wasn't until 2000 that it FDA-approved its current form for initial bladder cancer diagnosis as well as for surveillance [77]. A meta-analysis of studies of UroVysion FISH has calculated its sensitivity at 63% and specificity at 87% in the detection of bladder cancer [78].

Spectrum bias has also been reported for FISH [12]. Unsurprisingly, FISH sensitivity has been reported to vary by stage: pTa (65%), pTis (100%), and pT1-pT4 (95%) [79]. For surveillance, sensitivity was 55% (CI 36–72%) and specificity was 80% (CI 66–89%) [78]. When UroVysion is obtained in the context of an equivocal cytology, the reported sensitivity and specificity are 72% and 83%, respectively [80]. Importantly, several retrospective studies have noted that a persistently positive FISH result during Bacillus Calmette Guérin (BCG) therapy predicts a poor response to therapy [81–85]. If these results are validated in a current prospective trial, FISH could serve as an early indicator of BCG treatment failure.

FISH has also been shown to anticipate future bladder cancer [26, 27, 86]. These studies usually assume that any future bladder cancer that develops after a positive FISH can be attributed to the positive FISH test, even if it occurs years earlier. Others have disputed this claim and careful analysis has shown that only a portion of future bladder cancers are actually anticipated by FISH [21, 29, 59].

Perhaps the most common clinical utilization of FISH is to adjudicate positive or equivocal cytologies occurring in the context of a normal cystoscopy [87–90]. Multiple studies have shown that FISH detects most cancers and misses few high-grade bladder cancers when used in patients with equivocal cytologies [27, 89, 91, 92]. Furthermore, data from two prospective studies of reflex FISH testing (done in equivocal cytology or cystoscopy) showed a decrease in bladder cancer associated costs and a 60% PPV and 97% NPV [93].

Bladder Tumor Antigen (BTA) Tests

Bladder Tumor Antigen (BTA) test is a protein-based test that is FDA-approved for diagnosis and surveillance of bladder cancer. The BTA tests identify two basement membrane antigens, human complement factor H-related protein and complement factor H, which are present within the urine of bladder cancer patients [94]. The original BTA test described by Sarodsy and later validated by D'Hallewin [95, 96] was different than the current tests and is no longer available secondary to its low sensitivity and specificity [97]. There are now two forms of the BTA test available: BTA stat and BTA TRAK. BTA stat is a point of care test that uses an immunochromatographic method to give a result in 5 min and does not require specialized personnel [94]. A meta-analysis of 22 studies of BTA stat calculated the sensitivity as 64% and specificity as 77% [78]. This was confirmed in a second meta-analysis [98]. BTA TRAK is a quantitative sandwich immunoassay that requires a laboratory assessment and several hours to perform [99]. A meta-analysis of four studies of BTA TRAK calculated the sensitivity as 65% and specificity as 74% [78].

Overall, BTA appears to have a higher sensitivity but lower specificity than urine cytology [56, 98]. Like most urine tests, it does seem to anticipate future bladder cancer in some cases [95, 100–102]. The test suffers from cross reactivity with red blood cells since complement factor H is present in high concentrations in serum and consequently has a high rate of false positives in hematuria [103]. It also suffers from poor performance in patients treated with BCG due to local inflammation [104]. Studies of BTA tests suffer from poor reporting and [6, 33], consequently, test sensitivity has varied by study design, 66% in case-control studies and 77% in cohort studies [105].

Nuclear Matrix Protein-22 (NMP-22) Test

NMP-22 is an immunoassay that detects a nuclear matrix protein involved in the mitotic apparatus which is present in greater concentration within tumor cells [106–108]. NMP-22 has been FDA-approved for both diagnosis and surveillance of bladder cancer. Like BTA, NMP-22 is either available as a qualitative point-of-care test or as a quantitative, laboratory-based test. Meta-analysis estimated the sensitivity and specificity of the qualitative assay as 58% as 88%, respectively, and that of the

quantitative assay as 69% and 77% [78]. The improvement in sensitivity of NMP22 over cytology is due to improved detection of low-grade tumors.

NMP22 does, however, display spectrum bias. For example, the test has better sensitivity in women [107], and when multiple tumors are present [109, 110]. NMP22 anticipates future bladder cancers when cystoscopy is negative [111]. Several factors affect the performance of NMP22 including UTI, benign inflammatory conditions, urinary calculi, instrumentation, foreign bodies, other urologic malignancies, and genitourinary bowel interposition [112]. In fact, the false positive rate has been reported to be >80% when UTI is present and 100% with bowel interposition [113]. Even a concentrated urine secondary to dehydration can cause a false positive result by overestimating the NMP22 level [114]. In general, studies of NMP22 have been of poor quality [6, 33].

ImmunoCyt Test

ImmunoCyt is a cell-based test approved by the FDA for the bladder cancer surveillance. This test consists of fluorescent monoclonal antibodies that bind specifically to three cell surface glycoproteins present on the membrane of bladder cancer cells, making urinary bladder cancer cells visible microscopically. ImmunoCyt is used in conjunction with cytology to enhance the sensitivity of cytology [115–118]. A meta-analysis of 14 studies calculated the sensitivity of ImmunoCyt as 78% and specificity as 78% [78]. Due to spectrum bias, sensitivity increases with bladder cancer grade and stage. In a separate review examining the sensitivity, specificity, and predictive value of ImmunoCyt, the negative predictive value was better than the positive predictive value, suggesting that it has more false positives and fewer false negatives [119].

Perhaps the greatest limitation of ImmunoCyt is that, like cytology, the test is operator-dependent. Some investigators have found high interobserver variability and poor agreement [120], while others suggest that adequate training can overcome this limitation [121]. ImmunoCyt does not appear to anticipate future bladder cancers, though this aspect has not been carefully considered [122].

CxBladder Test

CxBladder is a cell-based test that identifies the presence of five mRNA fragments (MDK, HOXA13, CDC2, IGFBP5, CXCR2) in the urine that are expressed at high levels in patients with BC [123]. CxBladder is not FDA-approved though it is marketed for both hematuria evaluation and surveillance of BC. At a set specificity of 85%, CxBladder was able to detect 48%, 90%, and 100% of stage Ta, T1, and >T1 bladder cancers, respectively [123]. It was then validated in a cohort presenting with macroscopic hematuria [124, 125]. Based on a limited number of studies, test sensitivity is estimated to be ~85% and specificity ~85% [124, 126]. Given the paucity of studies involving Cxbladder, it is difficult to compare it to other urine-based diagnostic tests.

Breen et al. performed multiple imputations with five datasets to compare four diagnostic tests (cytology, NMP22, FISH and CxBladder) and found that Cxbladder had a higher signal-to-noise ratio and better sensitivity than the other tests [127].

Arguments for and Against Routine Urine-Based Testing for Bladder Cancer

The purpose of urine-based diagnostic tests for bladder cancer is ultimately to replace cystoscopy for hematuria evaluations or for bladder surveillance in patients with a history of bladder cancer. This is an excellent goal with potential significant benefit to the patient as well as healthcare costs. After all, cystoscopy is an invasive test that is expensive, impacts patient quality of life, and can cause adverse events like urethral strictures, pain, and urinary tract infections. Unfortunately, several limitations preclude the recommendation of routine urine-based testing in place of cystoscopy. In the case of hematuria evaluation, particularly microscopic hematuria, the pre-test prevalence of bladder cancer is so low (1–3%) that even a near perfect urine test would not change decision making. For example, in Table 8.4 we have calculated the pre-test probability of bladder cancer and the post-test probabilities of bladder cancer given either a positive or negative result on a urine test. This is actually an overly optimistic view because many of these tests can have indeterminate results which would complicate things further. What can be seen in Table 8.4 is that the none of the urine tests obtained for microhematuria, whether positive or negative, significantly change the probability of having BC and are therefore uninformative. In the case of gross hematuria, a negative test result is associated with a ~ 10% probability of having bladder cancer. Most patients and physicians would agree that a 1 in 10 chance of bladder cancer is high enough to proceed to cystoscopy. Therefore, the result of the urine test does not change the need for cystoscopy and is therefore of little utility.

A more complicated issue exists in non-muscle invasive bladder cancer (NMIBC) surveillance since the pre-test probability of disease depends on patient risk. This is related to the characteristics of their particular BC as well as the time interval between cystoscopies. In our BC population at Duke, for example, the 1-year probability of recurrence in patients with NMIBC undergoing surveillance is approximately 25%. This overall value is not personalized, however, and could be much higher or lower than what is seen in the general community due to referral and other biases. The EORTC risk tables can help in this regard [128], although they tend to overestimate risk slightly in modern cohorts that use immediate postoperative intravesical chemotherapy and second-look transurethral resection. For example, in low-risk NMIBC patients (EORTC score 0) the 1-year cumulative incidence of recurrence is 15%, and since these patients undergo annual cystoscopy the pre-test probability of having a tumor is also 15%. In the very high risk (EORTC score $\geq$ 10) cohort, the 1-year cumulative incidence of recurrence is 61%, but since these patients undergo cystoscopy every 3 months (at least initially), the pre-test probability of having a tumor is actually 21% (note that it is not 61%/4, the reasons for which are explained in the following reference [129]). In Table 8.5, we demonstrate how these factors

Table 8.4 Probability of having bladder cancer before and after a urine-based test done for hematuria

			Hematuria					
			Microscopic			Visible/Gross		
Test	Sensitivity (%)	Specificity (%)	Pre-test (%) [3]	Test positive (%)	Test negative (%)	Pre-test (%) [35]	Test positive (%)	Test negative (%)
Cytology [61]	37	95	1	7	1	18	62	13
UroVysion FISH [78]	63	87		5	0		52	9
NMP22 (quantitative) [78]	69	77		3	0		40	8
NMP22 (qualitative) [78]	58	88		5	0		51	9
BTA trak (quantitative) [78]	65	74		2	0		35	9
BTA trak (qualitative) [78]	64	77		3	0		38	9
ImmunoCyt [78]	78	78		3	0		44	6
CxBladder [78]	82	85		5	0		55	4

Table 8.5 Probability of having bladder cancer before and after a urine-based test done for bladder cancer surveillance

	EORTC recurrence score											
	Low risk (0)[a]			Intermediate (1–4)[b]			High (5–9)[c]			Very high (10–14)[d]		
	Pre-test (%)	Test positive (%)	Test negative (%)	Pre-test (%)	Test positive (%)	Test negative (%)	Pre-test (%)	Test positive (%)	Test negative (%)	Pre-test (%)	Test positive (%)	Test negative (%)
Cytology [61]	15	57	10	13	52	9	11	48	8	21	66	15
UroVysion FISH [78]		46	7		42	6		38	5		56	10
NMP22 (quantitative) [78]		35	7		31	6		28	5		44	10
NMP22 (qualitative) [78]		46	8		42	7		38	6		56	11
BTA trak (quantitative) [78]		31	8		27	7		24	6		40	11
BTA trak (qualitative) [78]		33	8		29	6		26	6		42	11
ImmunoCyt [78]		38	5		34	4		31	3		48	7
CxBladder [78]		49	4		45	3		41	3		59	5

[a]Assumes 1-year recurrence rate of 15% and cystoscopy done every 12 months
[b]Assumes 1-year recurrence rate of 24% and cystoscopy done every 6 months
[c]Assumes 1-year recurrence rate of 38% and cystoscopy done every 3 months
[d]Assumes 1-year recurrence rate of 61% and cystoscopy done every 3 months

affect test performance for NMIBC undergoing surveillance. We would argue that in all cases, any of the urine tests being positive would indicate a clear need for cystoscopy because even the worst performing test done with the most frequency would have a 24% probability of bladder cancer if positive. The more important question is whether a negative urine test would cause a clinician to forego cystoscopy. In some of the scenarios below, a negative urine test is associated with a < 5% risk of bladder cancer, which for some physicians and patients would be low enough to avoid cystoscopy. In other scenarios (very high risk), the risk with a negative test is still ~10% or so, probably more risk than most patients and physicians would accept to avoid cystoscopy.

Conclusions

Urine tests are widely available for bladder cancer, but their value in routine clinical practice is unclear. Careful consideration of how these tests affect clinical decision making is required in order to understand their use.

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Part II
Emerging Concepts in the Molecular Basis of Bladder Cancer Diagnosis and Therapy

Chapter 9
Molecular Targeted Therapy of Bladder Cancer

Elizabeth R. Kessler, Dan Theodorescu, and Thomas W. Flaig

Introduction

The urothelium extends from the renal pelvis to the prostatic urethra. Urothelial carcinoma represents the vast majority of cancers arising within the bladder. Bladder cancer is the ninth most common cancer worldwide [1] and will contribute to about 16, 390 deaths in the United States (US) in 2016. Nearly three quarters of all cases diagnosed are non-muscle invasive tumors; yet despite local treatment, there is a high rate of recurrence and in high-grade [2] tumors, progression to muscle invasive disease. This is a cancer of the elderly, as 90% of all bladder cancer patients are over 55 years old with a median age at diagnosis of 73 years old. The incidence is up to four times higher in men than women—likely due to tobacco use as a risk factor that occurs most often in men, although other factors may also play a role such as the androgen receptor [3]; despite these factors, bladder cancer does seem relatively more lethal in females [4]. This cancer is twice as common in white patients in comparison with African Americans or Hispanic Americans. However, black Americans have a higher bladder cancer mortality rate driven by a greater incidence of high-grade tumors [5]. Contributing to 50% of all bladder cancer cases, tobacco use is the most important risk factor for development of this condition [6]. Other risk factors include chronic urinary tract infection, or irritation. Occupational exposure to carcinogens such as aromatic amines and hydrocarbons is also viewed as a risk factor [7]. Some specific drug exposures such as cyclophosphamide (cancer chemotherapy) and phenacetin-based analgesics are also associated with urothelial

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carcinoma development [8]. These exposures produce an overall field effect in the urothelium that may spur multiple recurrences in the bladder as well as new primary tumors in other areas of the urinary tract given the common urothelial luminal epithelium. While not a clearly hereditary cancer, there are some emerging genetic risk factors as well as known genetic syndromes (i.e., Lynch Syndrome, Cowden disease, and retinoblastoma gene mutation) that are associated with the risk of developing bladder cancer [9, 10].

The medical treatment of metastatic bladder cancer has changed little in recent decades. This is reflected by 5-year survival rates as reported in the Surveillance, Epidemiology and End Results (SEER) database that have remained largely stable since the 1980s [11]. Through improved understanding of tumor immunology and bladder cancer genetics, new targeted therapy approaches are showing promise. This chemotherapy-sensitive tumor often responds initially to cytotoxic agents, but frequently relapses at a short interval, contributing to an overall survival for patients with metastatic disease of 9–15 months [12, 13] and median survival at relapse after first-line chemotherapy of approximately 6 months [14]. A newly approved immunotherapy agent for progressive metastatic disease, atezolizumab, is the first agent in decades approved by the Food and Drug Agency (FDA) for metastatic bladder cancer treatment.

For non-metastatic muscle-invasive cancers, therapy consists of either surgical resection with a radical cystectomy or definitive radiation therapy. The inclusion of cisplatin-based neoadjuvant chemotherapy offers both a survival benefit and decreased relapse rate [15]. Yet, the uptake of this approach has been limited to 10–20% of patients, in part due to provider perception that only a minority of patients benefit and the inability to safely administer chemotherapy in many patients due to tobacco-related comorbidities, and impairment of renal function [16]. However, new predictive biomarkers have the potential to overcome the overall limited benefit of neoadjuvant chemotherapy by selecting patients which could most benefit from the treatment. As the molecular understanding of bladder cancer improves, we have learned of its rich genetic variation and potential therapeutic targets as well as the potential for predictive markers to assign patients to their optimal therapy. This has led to a renewed interest in the treatment of this common cancer, and changed the management of bladder cancer.

Current Treatment for Advanced Bladder Cancer

Currently, cytotoxic chemotherapy is the foundation of metastatic bladder cancer therapy, reliant on platinum-based, combination therapy. These cisplatin-based regimens have been in use for decades with modest alteration. A common treatment regimen of methotrexate, vinblastine, adriamycin, and cisplatin (MVAC) has been shown to result in improved response rates and survival in comparison to single-agent cisplatin in the treatment of metastatic disease. The regimen is notable for significant hematologic, gastrointestinal, and infectious toxicities [17, 18] but a

dose-dense regimen has improved upon many of these toxicities and enhanced tolerability [19]. The dose-dense MVAC (ddMVAC) regimen shortens the treatment cycle from 4 to 2 weeks with mandated hematologic growth factor support. This approach allows for increased density of the cytotoxic drugs and results in an improved overall response rate (62 vs 50%) and complete response rate (31 vs 9%) in comparison to traditional 4-week MVAC dosing. While the median survival was similar between the standard versus dose-dense schedules (15.1 versus 14.9 months [mos]), the hazard ratio (HR) for survival did favor ddMVAC (0.76; 95% CI, 0.58–0.99), due to more long-term responses in the ddMVAC group. With 7 years of follow-up, 24.6% were alive in the dose-dense, versus 13.2% in the standard-dose MVAC arms [20]. As such, the dose-dense approach allows for increased dose density, lower toxicity when given in combination with growth factor support, and improved responses and subsequent survival when compared to the standard dosing and schedule. Another cisplatin-based regimen is the combination of cisplatin and gemcitabine (GC), which in the stage IV setting has been shown to have similar response rate to traditional MVAC (49 vs 45%) with a similar impact on survival (15.2 mos MVAC vs 14.0 mos with GC $p = 0.66$) [12] and has less high-grade hematologic toxicity. The GC regimen is in widespread use for advanced urothelial carcinoma as well and probably the most common regime used in the US [21].

There has been limited data to guide the medical treatment of patients with metastatic disease who progress on platinum-based therapy, without a traditionally approved chemotherapeutic option, and even lack of expert consensus on an appropriate regimen. The recent approval of atezolizumab, an immunotherapy agent directed at blockade of the programmed death ligand 1 (PD-L1), is the first second-line agent to garner FDA approval based on an improved overall response rate in comparison to historical controls [22]. Prior to the successful incorporation of immunotherapeutic agents into standard practice, multiple cytotoxic chemotherapies were tested with many utilizing a taxane-based approach in the second-line setting. Many of the molecular targeted options herein have been studied in combination with cytotoxic therapies that have historically been employed despite lack of robust results.

The ddMVAC and GC cisplatin-based regimens are also used in the neoadjuvant setting. The Southwest Oncology Group (SWOG) 8710 trial enrolled 317 subjects and randomized them to 3 cycles of standard-dose MVAC versus no chemotherapy and found an improvement in median survival in the chemotherapy arm (77 mos versus 44 mos $p = 0.06$) [15]. In addition, the rate of complete pathologic response to therapy at the time of cystectomy also improved in the arm receiving neoadjuvant MVAC from 15 to 38% ($p < 0.001$). A European-based study also evaluated the use of neoadjuvant chemotherapy in 976 subjects planning local treatment with either radiation therapy or surgery, and randomized them to a 21-day cycle of cisplatin-methotrexate-vinblastine (CMV) chemotherapy for 3 cycles versus no chemotherapy [23]. In those patients that underwent cystectomy, the pT0 rate, which correlates well with overall survival, was 32.5% in patients that received neoadjuvant chemotherapy versus 12.3% without. There has not been a large-scale trial of GC in the neoadjuvant setting but a single institution series compared GC to MVAC historical

controls and found a pT0 rate of 26% with GC compared to the historical control rate of 28% with MVAC [24]. Given its improved toxicity profile, and commonality amongst other cancer regimens, GC is widely utilized as a neoadjuvant regimen. However, despite these findings, neoadjuvant chemotherapy in general is not utilized as often as data would support. An epidemiologic analysis of the use of perioperative chemotherapy in patients with stage III (T3N0M0) bladder cancer reported that only 1.2% of these patients received neoadjuvant treatment [25]. Of those patients in this National Cancer Data Base analysis, 11.6% received either adjuvant or neoadjuvant, despite a lack of data supporting adjuvant chemotherapy. A recent retrospective analysis of Canadian patients also shows that while 19.4% of patients received adjuvant therapy only 3.1% received preoperative chemotherapy [26]. Smaller single-institution studies have reported similar rates in the US although one recent report indicated usage of 17% in a single-institution retrospective study [16, 25, 27]. Again, these statistics reflect that many eligible patients are not receiving neoadjuvant chemotherapy. Similarly, it is notable that of those patients that receive perioperative chemotherapy, it is more often given in the adjuvant setting—rather than neoadjuvant—despite the lack of robust randomized data supporting this approach. It likely relates to the finding that less than 10% of patients treated with neoadjuvant chemotherapy benefit [15] while finding nodal metastases at cystectomy is generally perceived as a strong reason to treat adjuvantly despite the lack of definitive data supporting benefit. While the driving cause of lower adoption of neoadjuvant chemotherapy is unclear, lack of neoadjuvant treatment remains a significant barrier in improving the disease-specific outcomes for patients with muscle-invasive bladder cancer.

Molecular Targets and Targeted Therapy in Bladder Cancer

Across the field of oncology, treatment selection has increasingly been informed by markers predictive of response to therapy or prognostication of disease course, as such the therapeutic plan is regularly driven by molecular data. For example, breast cancer and lung cancer therapies have long been built around epidermal growth factor receptor 2 (HER2/neu) or epidermal growth factor receptor 1 (EGFR) mutational status, respectively. There are now approximately 15 recognized molecular subtypes of lung cancer, each with an associated therapeutic agent either approved or under investigation. Oncologic treatment in these diseases is now guided by both molecular and histologic classification. In other cancer settings, treatment has also expanded to include the use of targeted therapies without a clear biomarker correlate. For example, the targeted inhibition of the vascular growth pathways has proven successful in multiple tumor types without any identified predictive biomarker.

Increasingly, genomic data has revealed 3–4 bladder cancer subtypes based on clustered alterations in urothelial carcinoma, which can form patterns or groupings with predictive and prognostic implication (Table 9.1). However, the success of biomarker-driven treatment mirroring other tumor types has not yet been realized in

Table 9.1 Molecular subtypes of bladder cancer

Molecular subtype	Describing group and associated alterations				
	UNC{Damrauer, 2014 #187}	MDACC {Choi, 2014 #61}	TCGA{2014 #128}	Tumor initiation differentiation{Chan, 2009 #146}	Clinical correlation
Basal-like	KRT5 KRT6 CD44	KRT5 KRT14 CDH3 CD44	Type I–papillary histology FGFR3	KRT14 CD44 (some overlap with group III, IV or the TCGA)	May have reduced survival outcomes More advanced presentation
Luminal-like	PPARG GATA3 KRT20 UPK2 FGFR3 TSK1	KRT20 CD24 FOXA1 GATA3 HER2 ERBB3	Type II—HER2, estrogen receptor	KRT8 KRT18 KRT20	Suggested improved immunotherapy response
Other clusters		P53-like	Type III–combined with SCC head and neck and lung, elevated KRT14, CD44 Type IV–like cluster III with surrounding muscle	Differentiated: KRT5 KRT17 CD44	

UNC University of North Carolina, *MDACC* MD Anderson Cancer Center, *TCGA* Tissue Cancer Genome Atlas, *KRT* Keratin, *CD* cluster of differentiation, *FGFR3* fibroblast growth factor receptor 3, *PPARG* peroxisome proliferator-activated receptor gamma, *UPK2* uroplakin2, *TSK1* tight skin 1, *FOXA1* forkhead box A1, *SCC* squamous cell carcinoma

the treatment of bladder carcinoma. Historically, the attempt to target various molecular alterations in bladder cancer tumors has not resulted in positive clinical outcomes for patients, but this area is rapidly evolving based on new data.

Molecular Basis of Bladder Cancer

Vascular endothelial growth factor (VEGFR), HER2, Programmed Death (PD), and FGFR3 have been explored as therapeutic targets with varied success in large-scale trials (Table 9.2). Recently, large-scale genomic analyses such as the Cancer Genome Atlas (TCGA) data offer insights into relevant pathways leading to interest in new therapeutic targets, which will need further validation. This increased biologic knowledge may in turn allow for a more precise understanding of the progression and treatment of bladder cancer. A summary of TCGA results is described in detail in Chap. 3 of this text.

Table 9.2 Important pathways in bladder cancer therapy

Pathway	Treatment method	Agents	Current status of clinical development
FGF	Tyrosine kinase inhibitor	Divotinib [28]	Phase II
HER2	Monoclonal antibodies	Trastuzumab [29]	Phase II
	Tyrosine kinase inhibitor	Lapatinib [30]	Phase II
	Immune modulation	DN24–02	Phase II (NCT01353222)
Immune checkpoint inhibition	Monoclonal antibody	Atezolizumab [21]	Phase II—FDA approved
		Pembrolizumab [31]	Phase Ib, phase III - FDA approved
		Durvalumab and tremelimumab	Ongoing phase III study (NCT02516241); single agent appro val for durvalumab
		Nivolumab with or without Ipilimumab	Phase I (NCT02496208); ; Phase II single agent nivolumab - FDA approved
VEGF	Monoclonal antibody	Bevacizumab [32]	Phase II, ongoing phase III
	Tyrosine kinase inhibitor	Vandetanib [33]	Phase II
		Ramucirumab [34]	Phase II, ongoing phase III
		Pazopanib [35, 36]	Phase II
		Cabozantanib	Ongoing phase II
		Sunitinib [37, 38]	Phase II
		Sorafenib [39]	Phase II

FGF fibroblast growth factor, *VEGF* vascular endothelial growth factor

Bladder Cancer Subtypes

Interestingly, analysis of these data allowed for a separation of bladder cancer into two main subtypes (luminal and basal) which were then divided into four clusters: cluster I and II (luminal) and cluster III and IV (basal). The basal cluster III is similar to squamous cell cancers and the luminal cluster I is papillary-like. While there is a need for validation through carefully designed clinical trials, this genomic analysis offers insight into the molecular makeup of urothelial carcinoma and prognostic/predictive factors. As noted, the high mutational load places bladder cancer in the company of lung cancer and melanoma, further bolstering the historical translational relevance of lung cancer therapeutics in bladder cancer and suggesting that the relevance of immunomodulation in these cancers may also be similar. There is a rich amount of data in the TCGA set and while some of the identified targets have been explored without clear success thus far, these TCGA data also direct our attention to previous unstudied pathways (i.e., chromatin regulatory genes) and inform a future path of targeted therapies in this field.

Another approach has focused on classifying cancer types according to "cell of origin" commonalities [40]. Twelve cancer types were analyzed and organized into clusters according to a molecular taxonomy. Most of the cancers still settled amongst

histologic commonality but some molecular features did create unique subtypes. Bladder cancer as a whole was split into three major subtypes: the C1-LUAD (lung adenocarcinoma)-enriched, the C2-squamous-like, and the C8-BLCA (bladder cancer) group. The majority of the bladder cancer cases in the squamous-like group contained some degree of squamous differentiation, but urothelial carcinoma was still the dominant histology in most cases. This group included squamous cell lung, head and neck, and bladder cancers. This subtype was characterized by alterations in p53, amplification in p63, and high expression of genes within pathways related to immune function and proliferation. These included the PD1 signaling pathway and CTLA4 pathway; or proliferation genes such as cMYC and the FOXM1 signaling pathway. There were clear differences between the two largest bladder cancer groups (the C8-BLCA and C2-squamous-like), the C2 group showed a pattern of 3p loss (as expected in a squamous cancer) whereas the C8 group retained 3p. Also of interest, the C2-squamous-like group demonstrated higher levels of immune cell-associated signatures. It will be important to explore this finding further, as we look at responders to newer immunotherapies, including programmed death 1 (PD1) and PD-L1 inhibitors, and work to validate these findings. The C8-BLCA group had improved survival in comparison to the other two bladder cancer subtypes and may serve to prognosticate a course for patients that may require altered treatment. Overall, the creation and clinical associations of these subtypes further support the characterization of cancers by integrating histologic information with molecular features which may be predictive of response to therapy or disease biology.

Investigators have looked into the additional molecular classification schemas for bladder cancer tumors in an effort to inform treatment and prognostic areas [41]. An additional approach has been to classify bladder cancer tumors into three subtypes predictive of tumor initiation patterns based on an analysis of their differentiation states. It is well understood that bladder cancers arise from the urothelium which consists of basal, intermediate, and umbrella cells, each representing increasingly differentiated states, respectively. The authors evaluated the molecular characteristics of these three states. Basal cells are characterized by coexpression of CD44 and Keratin (KRT) 5, whereas terminally differentiated cells (such as umbrella cells) will express KRT20 but not CD44 [42]. Using this knowledge as a starting point, analysis of the differentiation and tumor initiation of bladder carcinoma was undertaken as another means of disease characterization. A computational model was developed to associate cell surface markers that correspond with bladder cancer differentiation stages through analysis of gene-expression databases [41]. Tumor cell populations possessing marker profiles corresponding to discrete differentiation states were then transplanted into mice to interrogate their effect on the growth of tumors. Further refined analysis subsequently correlated with archival tissue data sets to identify a link between these markers and clinical outcomes. The basal subtype was associated with worse overall survival compared to both the intermediate and differentiated subtypes. This was validated within two additional data sets and is likely weighted by the expression of KRT14. Using these differentiation groupings, if validated in a prospective manner, one may evaluate cell surface markers, classify tumors, and perhaps understand more of the prognostic nature of a particular bladder cancer tumor.

This sub-classification of bladder cancer has also been proposed by other groups characterizing muscle-invasive bladder cancer as basal, luminal, or p53-like [43]. Through whole genome mRNA profiling of 73 primary muscle-invasive bladder tumors, these three molecular subtypes emerged. These types have similar genetic changes to those seen in basal and luminal breast cancer with the following profiles of upregulated genes: basal—CD44, KRT5, KRT6, KRT14, CDH3; luminal—CD23, FOXA1, GATA3, ERBB2, ERBB3, XBP1, and KRT20. A third type, the p53-like, is very similar to the luminal breast cancer grouping but also possessed activated wild-type p53 expression. The luminal subtype exhibited peroxisome proliferator activator receptor (PPAR) activation whereas the basal subtype was more likely to have p63 activation. In retrospective datasets, these subtypes have correlated with clinical outcomes with the basal tumors more highly associated with shorter overall survival, squamous differentiation, and advanced disease at presentation. The p53-like group correlated with clinical resistance to cisplatin-based chemotherapy with an overall response rate of 11% in tumor samples analyzed from patients participating in a small phase II trial of neoadjuvant chemotherapy [43]. While the approach to grouping these tumor-types differ, with varied classification overall, and suggest that there may be some utility in looking beyond histologic characteristics and understanding more of the molecular make-up of bladder cancer in order to improve our understanding of the disease biology.

Predictive Biomarker Models

Predictive biomarkers allow clinicians to select a therapy, targeted at a genetic alteration, with the knowledge that the presence of the alteration predicts for response to the treatment. For example, presence of an anaplastic lymphoma kinase (ALK) oncogene rearrangement predicts for response to crizotinib in non-small cell lung carcinoma and its presence is used to select patients for that treatment. Many clinically-utilized predictive biomarkers are matched with targeted therapies that target specific pathway dependence or discrete mutations. However, treatment selection oftentimes is more complicated than matching a detected mutation to the corresponding targeted therapy. Additionally, not every mutation is necessary and sufficient for cancer growth and different cancer types may have different responses to a specific mutation with respect to its primary or driver status. It is also notable that, while predictive biomarkers have demonstrated increased uptake in regards to targeted therapeutics, it must not be forgotten that biomarkers may also predict for response or resistance to cytotoxic chemotherapy. At this time, cytotoxics are the mainstay of the treatment of advanced bladder cancer and will likely remain a cornerstone of therapy for the foreseeable future. Despite this, there are more limited clinical data predicting response to particular active agents.

COXEN

As mentioned, there are predictors of response to chemotherapy in development, with the CO-eXpression ExtrapolatioN (COXEN) model as one leading example; a gene-expression model which may be used to predict an individual patient's cancer's response to specific agents including cytotoxic drugs. COXEN translates cell line drug sensitivity with gene expression as an initial step. The NCI-60 is a well-studied group of 60 cell lines from nine common cancers; these lines have been tested with a multitude of agents and assessed for both drug sensitivity and gene expression [44]. The COXEN model utilizes the gene expression signature of the NCI-60 cell lines and their response to specific agents, and then correlates these findings with the gene expression profile of patients in specific settings, to identify concordant genes [45]. This process identifies clinically relevant genes and "humanizes" the in vitro data. These data are then used to calculate a COXEN score for a single drug, or derive a score for a combination of drugs. The combination COXEN score is a probability based on 5-year survival data, which allows for a calculated correlation coefficient (COXEN coefficient) that may then be used to predict drug sensitivity through this generated gene expression model. This methodology has been applied retrospectively to data from two small trials [46, 47] yielding significant prediction of responders and non-responders based on COXEN scores alone. In one analysis, gene expression profiling was performed to determine response in the locally advanced or metastatic setting predicting "responders" and "non-responders" with significant differences seen in the COXEN score that correlated with 3-year survival rates in bladder cancer [46, 48]. In another setting, COXEN was applied to patient data in the neoadjuvant setting and a significant difference in the COXEN scores of those with down staging and those without a pathologic response was observed [47]. The COXEN method is now in study in a randomized phase II National Clinical Trials Network SWOG study (S1314) of patients with muscle-invasive bladder cancer. In the clinical trial, patients are randomly assigned neoadjuvant chemotherapy with ddMVAC or GC. Through extensive modeling, scores were derived for both regimens in the S1314 study and validated using a combined bladder cancer tumor cohort consisting of two datasets (n = 278). If the study is found to be feasible, and the COXEN score associate with pathologic complete response, the results of this trial will be an essential part of validating this model for clinical use in bladder cancer. In bladder cancer, the NCI-60, BLA-40, and GDSC-648 cell panels were used to identify predictive biomarkers for sensitivity to the drugs in the GC and MVAC regiments. Predicting responders to a particular therapy, and certainly predicting responders to neoadjuvant therapy may then improve the uptake of this treatment, and allow those with a low predicted likelihood of response to proceed to surgery without delay,

and for those with high likelihood of response to be treated with the most effective chemotherapeutic combination.

Single Nucleotide Polymorphisms (SNP)

Germline alterations have long been known to be of importance to the metabolism of pharmacologics—subsequently influencing both tolerance and response to therapy based on the drug level differences seen with the alteration. In bladder cancer, germline single nucleotide polymorphism (SNP) markers have been evaluated as predictors of response to cytotoxic chemotherapy. For example, there have been investigations identifying potentially important polymorphisms in genes that may affect cisplatin sensitivity [49]. The identification of a predictive biomarker for response to pre-operative chemotherapy could increase acceptance of this approach and minimize toxicity and surgical delay for those patients unlikely to respond. In a study of SNPs as predictors to chemotherapeutic response, patients undergoing treatment with neoadjuvant cisplatin-based chemotherapy provided a germline DNA sample. Of these samples, nine SNPs were selected based on their association with platinum-sensitivity in pre-clinical studies and the observed rate of complete response to platinum-based chemotherapy at cystectomy (pT0 rate). These nine germline single nucleotide polymorphisms were tested retrospectively in a large cohort of 205 patients with bladder cancer through a discovery and validation set approach. Three SNPs emerged as potentially correlated with pT0 and <pT2 rates, but these were not replicated in this independent data set [50]. While this analysis did not validate this set of SNPs as a predictive marker of bladder cancer chemotherapeutic sensitivity, we have learned that in other disease settings the general use of SNPs for prediction of drug response has promise.

Clinical Investigations of Targeted Therapy in Bladder Cancer

P53

There are natural checkpoints in the cell cycle that allow for DNA repair, cell cycle arrest, or cell death. The p53 gene plays a key role in cell cycle arrest, apoptosis and cellular senescence [51, 52]. As the most frequently mutated human tumor suppressor gene, p53 downregulation allows for continued accumulation of cellular mutations and tumor growth. In bladder cancer, more than 50% of muscle-invasive tumors contain alterations in p53, and it is hypothesized that these play a role in the progression of urothelial carcinoma [53, 54]. These p53 mutations are also prognostic, as p53 status was the strongest predictor of disease specific outcomes in a small series of patients with invasive bladder cancer treated with cystectomy [55], and in

a series of patients treated with neoadjuvant MVAC [56]. In addition to prognostication, p53 may predict response to MVAC [54]. Based on these data, a prospective phase III study of adjuvant therapy for locally advanced bladder cancer based on p53 status was undertaken to validate the utility of p53 status [57]. Patients with p53 mutation were randomized to receive 3 cycles of MVAC chemotherapy versus observation. All patients with p53 negative status, as tested via IHC, were observed. Of the 499 patients assessed for p53 status, 55% were positive. Unfortunately, despite a large effort to screen hundreds of subjects, this study closed early due to futility with only a small fraction of subjects entering randomization and completing MVAC chemotherapy as assigned—39 of 499 evaluated patients—with no difference in the observed recurrence rates between the two treatment groups of chemotherapy versus observation. A high rate of patients participating in this trial refused randomization or did not complete therapy, additionally this endeavor failed to support the prognostic value of p53.

HER2

The human epidermal receptor (HER) family is composed of four receptor tyrosine kinases: HER1 (Erb-B1 or EGFR), HER2 (Erb-B2 or Neu), HER3 (Erb-B3), and HER4 (Erb-B4). The HER2 transmembrane tyrosine kinase receptor has no known natural ligand but instead acts through dimerization with other members of the HER family to activate multiple cell survival pathways [58]. Agents targeting HER2/neu, such as trastuzumab, are part of standard-of-care therapy in HER2/neu-positive breast cancer and HER2/neu over-expressing gastric cancer. In urothelial cancers, there are notable amplifications and mutations in this receptor with the TCGA data reporting 9% frequency [59] and some clinical series reporting as high as 45% of urothelial carcinoma samples with protein overexpression [60]. These alterations have been targeted through various therapeutic approaches.

Several small studies have investigated the use of HER2 inhibitors in bladder cancer. Notably, a single arm phase II trial investigated the safety and efficacy of trastuzumab in combination with gemcitabine, carboplatin, and paclitaxel [29]. Patients with metastatic urothelial carcinoma and HER2 overexpression by any of three methods were included: immunohistochemistry (IHC), serology, or fluorescent in situ hybridization (FISH). This study enrolled 57 patients and 44 went on to receive therapy for a median number of 6 cycles, resulting in a 70% response rate with five complete responses. The authors reported a median overall survival (OS) of 14 months and median time to progression (TTP) of 9.3 months, which compare favorably with disease control rates seen with GC use in this setting. Results from this study suggested an increased response to treatment in direct correlation with IHC expression of HER2. Given the small sample size and the lack of a control arm to address an overall prognostic implication of HER2 positive urothelial carcinoma, the true contribution of trastuzumab in this setting is unclear, but the report of many complete responses is notable. Another trial of patients with metastatic UC studied the use of lapatinib versus placebo as maintenance therapy

after first line platinum-based chemotherapy [30]. Patients with HER1/2 positivity by IHC, who achieved clinical benefit after first-line chemotherapy, were eligible to be randomized to the HER2 inhibitor, lapatinib, or placebo. Of the 455 patients screened, 232 were positive for HER2, and randomized. There was no statistically significant difference between the progression-free survival (PFS) or OS of the two treatment groups.

Another approach to targeting HER2 was tested in the adjuvant setting through the use of an immune targeted therapy. The agent, DN24–02, is an autologous cellular immunotherapy, similar to Sipuleucel-T therapy approved for prostate cancer, but is directed at HER2 in this case. In this treatment strategy, patient specific antigen presenting cells (APCs) were cultured with BA7072 (a HER2 derived antigen linked to granulocyte-macrophage colony stimulating factor) and then infused back into the patient on three separate occasions. DN24–02 was investigated in the NeuACT study (NCT01353222), which enrolled patients with ≥pT2 node or pathologic node positive urothelial carcinoma of the bladder or ureter at the time of surgical resection. Patients with HER2 expression of at least 1+ by IHC were randomized to undergo 3 cycles of leukapheresis and infusion of the DN24–02 autologous cellular product versus observation. An interim report of 226 patients reported the positive IHC rate based on the primary tumor site of 75% of submitted samples expressing HER2 of at least 1+ in the primary tumor compared to 84% in the dissected pelvic lymph nodes [61]. Appropriate humoral immune responses to infusion were observed in the subjects, but disease activity is unreported at this time. It is hoped that this large trial will contribute randomized data addressing the utility of HER2 directed therapy. As seen in the NeuACT study, most bladder cancers express HER2 to some degree, and as such, successful targeting of this receptor could have a broad impact on bladder cancer treatment. HER2 remains an interesting therapeutic target based on its alteration in urothelial carcinoma and utility in other disease settings, and a definitive trial has yet to be accomplished.

VEGFR

Angiogenesis has long been regarded as a natural target in cancer therapy—the approach is conceptualized as depriving a nest of tumor cells of the vascular network necessary to deliver nutrients with this approach proving successful in several disease settings within oncology. Vascular endothelial growth factor receptors (VEGFR) 1 and 2 and their ligands (including VEGF-A, -B, -C, and -D) are important mediators of tumor angiogenesis and likely contribute to the pathogenesis and progression of bladder cancer [62, 63]. While preclinical study has shown a noted impact on tumor growth and metastatic potential through inhibition of angiogenesis, it has been difficult to translate these preclinical observations into successful clinical gains. And yet, we have seen meaningful contributions of anti-angiogenic agents in the treatment of multiple tumor types. Certainly, highly vascular tumors such as renal cell carcinoma and hepatocellular carcinoma have recognized the

greatest gains clinically (by using anti-angiogenic agents as monotherapy), but tumors such as urothelial carcinoma have also been shown to respond to these agents. In general, VEGFR has been targeted through tyrosine kinase inhibitors and humanized monoclonal antibodies. Agents directed at vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are approved in the treatment of colorectal cancer, non-small cell lung cancer, glioblastoma, breast cancer, ovarian cancer, hepatocellular cancer, and renal cancer. The following section will review the investigation of anti-VEGF therapies in the treatment of urothelial carcinoma.

Bevacizumab

Bevacizumab is a humanized monoclonal antibody against VEGF-A, which has been successfully shown to improve outcomes in several oncology settings including improved survival in lung cancer, colorectal cancer, and progression-free survival in breast cancer [64–66]. A phase II, single-arm trial by the Hoosier Oncology Group investigated the activity of cisplatin, gemcitabine, and bevacizumab in patients with previously untreated metastatic or locally advanced bladder cancer [32]. In this trial, there was an overall response rate (ORR) of 72% and progression free survival of 8.2 months with an overall survival of 19.1 months. These data are encouraging compared to outcomes seen with standard platinum-based therapies. To more definitively distinguish the role of bevacizumab in the metastatic setting, a multi-center, randomized, phase III trial (CALGB 90601) of 500 patients is comparing 6 cycles of GC to GC+ bevacizumab (NCT00941331). Patients in the GC+ bevacizumab arm received bevacizumab every 3 weeks with GC and then continued on a maintenance treatment with bevacizumab until disease progression or unacceptable toxicity. This trial is fully accrued with results pending.

Vandetanib

Both VEGFR and EGFR have shown biologic relevance in the development and progression of urothelial carcinoma [67]. In addition, the identification of anti-cancer activity preclinically with the blockade of these receptors led to the exploration of tyrosine kinase inhibition of these pathways in the treatment of bladder cancer [68, 69]. Vandetanib is a tyrosine kinase inhibitor targeting VEGFR2 and EGFR. A phase II trial assessed the contribution of vandetanib in combination with docetaxel in treating patients with urothelial carcinoma who had previously received platinum-based chemotherapy [33]. One hundred and forty-two patients were enrolled and randomized to docetaxel plus placebo or docetaxel plus vandetanib. Until recently, taxanes had been among the most commonly utilized treatment options in the second-line setting, and represented a standard of care treatment in

this study [70]. The median PFS in the combination arm was 2.56 months in comparison to 1.58 months in the docetaxel arm (HR 1.01 95% CI 0.69–1.49; $p = 0.939$). The overall response rates (7% in the combination arm versus 11% in the docetaxel arm $p = 0.56$) and median overall survival were similar in both arms (5.85 versus 7.03 months, respectively with a HR of 1.21; 95% CI 0.81–1.79; $p = 0.347$). Of note, one patient did achieve a complete response when treated with docetaxel and vandetanib, but this trial failed to show an overall clinical benefit for patients treated with the VEGF/EGFR inhibitor in comparison to single-agent docetaxel.

Ramucirumab

Bevacizumab has been the most widely utilized agent to target the VEGF/VEGFR pathway in oncology. Newer targeted VEGFR antibodies, such as ramucirumab, have been developed with high specificity for VEGFR-2 and are able to outcompete VEGF-A, -C, and -D for binding to the receptor [71]. Icrucumab also targets the VEGF-1 subtype of VEGFR, and demonstrated high specificity and affinity in early investigations [72]. A phase II, three arm, trial compared docetaxel monotherapy versus combinations with the targeted agents, ramucirumab or icrucumab, in the second-line setting for treatment of advanced bladder cancer [34]. This study randomized 148 patients to these three treatment arms and found that patients treated with docetaxel in combination with ramucirumab experienced a significantly improved PFS of 5.4 months as compared to 2.8 months in the docetaxel-alone arm ($p = 0.0002$). This combination group also completed the most cycles of chemotherapy (median 4.5) and had the longest "on therapy" duration (median duration 14.3 weeks compared to 9.1 weeks in docetaxel-alone and 7.0 weeks in docetaxel plus icrucumab arms). The overall survival difference in these groups was not statistically significant, but the extension of PFS is noteworthy given the lack of large-scale benefit of VEGFR agents in bladder cancer thus far. A larger international phase III trial of docetaxel and placebo versus docetaxel and ramucirumab in patients with metastatic urothelial carcinoma who have progressed on platinum-based chemotherapy was subsequently launched to investigate the role of ramucirumab (NCT02426125).

Additional early phase studies targeting VEGF: There are multiple small molecule inhibitors targeting the VEGF receptor or otherwise altering the functionality of this vascular endothelial growth pathway. Many of these are specific for VEGF but others target a broader kinase group. Early preclinical studies have suggested activity in targeting this pathway, but the few completed small phase II trials have failed to show improved overall outcomes. One such multi-kinase VEGF inhibitor, pazopanib, had showed promise in preclinical examinations [35] and was studied in combination with docetaxel or versus docetaxel alone in later-line metastatic UC patients with mild improvement in PFS, but no significant improvement in OS or ORR [33]. The combination of vandetanib with paclitaxel was assessed in a small phase II trial ($n = 32$) with an encouraging response rate of 50% (11% complete

response) [36]. However, the combination also resulted in significant myelosuppression requiring growth factor support in almost half of the patients. The median overall survival of patients in the study was 8 months with median PFS of 6 months. Another, multi-kinase inhibitor with primary VEGFR activity, cabozantanib, is also being explored in this setting as this drug has been approved for use in thyroid and renal cell cancers, and targets hepatocyte growth factor receptor (MET) in addition to VEGFR. Results of this treatment used alone or in combination with nivolumab in bladder cancer are still pending (NCT02496208). Early clinical investigations with cellular models of bladder cancer have been undertaken with sunitinib, which primarily targets the VEGF and platelet-derived growth factor (PDGF) pathways. In a phase II trial of sunitinib as first-line therapy for metastatic cisplatin-ineligible UC patients, a median overall survival of 8.1 months was observed with sunitinib monotherapy, which is less than that seen with carboplatin-based chemotherapy in this setting [37]. A separate phase II trial in patients with metastatic disease previously treated with medical therapy revealed a median overall survival of 7.1 months with sunitinib [38]. Sorafenib is another multi-targeted small-molecule TKI that targets the VEGFR, but similar to sunitinib, it targets several other tyrosine kinase receptors beyond VEGFR. When used as monotherapy in the metastatic urothelial carcinoma setting, there has not been any signal of clear clinical activity [39]. Other novel manipulations of angiogenesis through angiopoietin-Tie2 signaling inhibition or fibroblast growth factor receptor inhibition are also under investigation. The modulation of the VEGF pathway reveals some early promise, but no large-scale results to date.

FGFR

Fibroblast growth factor pathway FGFR mutation is found in 5–15% of urothelial carcinomas with fusions of FGFR found in 3–5% as evidenced through next generation sequencing with protein overexpression found in 85% of mutated tumors and 42% of non-mutant tumors [73, 74]. This receptor is important in overall tumorigenesis and is a component of angiogenesis regulation. Due to the notable frequency of FGFR abnormality observed, this pathway has been explored as a potential therapeutic target, although it is not clear how often FGFR serves as a "driver" for growth and development of advanced bladder cancer, or how to appropriately assess a predictive biomarker (i.e., mutation or upregulation of the FGFR). A clinical trial of dovitinib, an oral inhibitor of FGFR3 and other proangiogenic receptor tyrosine kinases, failed to reach its primary endpoint of overall response rate in a Simon's two stage designed trial [28]. Patients with advanced urothelial carcinoma received divotinib, regardless of FGFR mutational status, in the later-line setting. Forty-four patients were treated with 12 patients possessing FGFR3 mutation and 31 with wild type FGFR3. The ORR in the trial was 0% in the mutated group and 3% (one patient with a partial response) in the non-mutated group. This study did not proceed to the second stage of the two-stage design due to low response rates. It is not clear if this

lack of clinical efficacy is due to an irrelevance of the pathway or inadequate target inhibition as divotinib primarily inhibits VEGFR and may have had limited specificity for the FGFR. In addition, the FGFR mutation assay was not particularly sensitive, and thus the selected population was not truly reflective the mutational rate within a larger bladder cancer population.

Eribulin

While much of the current therapeutic focus is on targeting various important cell signaling or immunomodulating pathways, there is still a role for cytotoxic therapy. Eribulin is a novel cytotoxic agent derived from sea sponges, targets microtubule function within the cell and is derived similar to taxane therapy and has been evaluated in UC. A phase II study of 44 patients with urothelial carcinoma and previous platinum therapy were treated with eribulin [75]. Of 37 evaluable patients, there were nine partial responses and two complete responses [75]. This promising response rate warrants further investigation. This agent has also been investigated in the first line setting for the treatment of advanced urothelial carcinoma in a small Phase Ib/II study [75, 76]. Nine patients were enrolled into the phase I portion and six into the phase II; with an overall response rate in the phase I portion of 89% including two complete responses. Based on these responses, a larger multi-center study enrolled 150 patients with advanced bladder cancer. This trial studied patients in the first or second line, and included patients with previous taxane exposure. The trial employed a Simon's two-stage design with a required response rate of greater than 20% for a cohort to enroll additional patients, and all cohorts have met this response and have continued to enroll accordingly [75].

Immunotherapy

Immunotherapy directed at CTLA4 and PD1/PD-L1 has forever changed our approach to the treatment of cancer. While immune checkpoint inhibition was initially found to be active in melanoma and renal cell cancers, this approach has shown particular promise in urothelial carcinoma. Bladder cancer has a long history of immunosensitivity based on the effectiveness of intravesical instillation of Bacillus Calmette-Guerin (BCG) in the treatment of superficial urothelial carcinomas since the 1970s. Early-phase trials of PD1/PD-L1 inhibition have shown a response rate comparable to- or better than- currently accepted therapies. While the first-line treatment for urothelial carcinoma has long been a platinum-based combination regimen, no one second-line chemotherapy has shown a clear survival benefit, and there is no current consensus as to the preferred second-line chemotherapy. Recent study of atezolizumab in patients with advanced urothelial carcinoma after platinum chemotherapy has revealed significant activity, including prolonged durability of responses, and garnered approval by the FDA for UC in

2016. The PD-L1 pathway and ongoing clinical trials are covered in the subsequent chapter.

Conclusions

While urothelial carcinomas may possess a large number of genetic alterations suitable for targeting with treatment in comparison to other cancers, no targeted therapy based on a specific driver mutation is currently used in clinical practice. Recent work has begun to use these data to rationally sort and group urothelial carcinoma with the goal of aiding in drug selection and prognostic determination. Clinical trials investigating the effectiveness of targeted many of these altered pathways have suggested some activity. Several promising targeted therapies are in late-stage testing with inhibition of the VEGF pathway notably among them. Moving from manipulation of cell-signaling pathways, the next era in medical oncology will likely include further modulation of the immune system response to tumors. Atezolizumab is the first FDA-approved urothelial carcinoma agent in the contemporary era and has initiated renewed interest in bladder cancer therapeutics. As we learn more about the tumor-directed immune response, we will perhaps be able to prospectively use mutation load or TCGA categorical profiles to predict for response and aid in patient selection beyond the PD1/PD-L1 testing in development. The reliance on platinum-based chemotherapy as the foundation of our medical therapy approach is shifting to include more molecular data in the treatment of bladder cancer as well.

Future Directions

As we assess the current status of molecular therapies in bladder cancer, it seems that the field is on the cusp of making some meaningful breakthroughs based on improved biologic insights, biomarker validation, and new targeted therapies. It has taken decades to arrive at this point, but there is a sense of rejuvenation in the drug development field for this prevalent cancer. Perhaps the scientific attention and momentum will carry over to the clinical care of patients with bladder cancer. In that way, we may see increased enrollment of patients on clinical trials, increased data from which will help to better understand the biologic characteristics of the disease, and as such an improvement in the overall approach to the treatment of bladder cancer. The treatment of lung cancer exemplifies the use of molecular data to both prognosticate and predict response to targeted therapies, and serves as a hopeful future model for urothelial carcinoma. Cancer research has focused not only on identifying mutations, but then characterizing those that are necessary for disease progression. This approach can be similarly employed in bladder cancer with an expanding biologic understanding of the disease. The use of predictive

models, such as COXEN, may aid in choosing the proper therapy for patients. As we begin to utilize biomarkers and predictive models, we may be able to improve upon our assessment and planning of treatment options for patients ineligible to receive cisplatin based on either organ dysfunction or performance status restrictions. Through genomic analysis it appears that bladder cancer has a diverse and large number of genetic alterations within the tumor. Many of these are yet to be validated, but clinical development has focused on the vascular and immune systems thus far and show great promise as potential therapeutic targets. Open questions in optimizing medical therapy include defining the duration of treatment needed with immunotherapy, or the use of PD1/PDL1 inhibition in the adjuvant setting are to be answered in further clinical investigation. The field is working to answer many outstanding questions with the aspirations of improving outcomes for patients through leveraging the improved biologic understanding of the diverse molecular landscape in urothelial carcinoma.

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Chapter 10
Response to Immunotherapy: Application of Molecular Pathology to Predict Successful Response

Noah M. Hahn and Donna E. Hansel

Introduction

The FDA approval of the PD-L1 monoclonal antibody atezolizumab for the treatment of post-platinum metastatic urothelial carcinoma (UCa) patients in 2016 marked the first systemic therapy approval for UCa in three decades [1]. This sentinel event has sparked a renewed optimism for further improvements in clinical outcomes through the incorporation of immunotherapy into current and future treatment approaches. However, this justified optimism is tempered by the challenges of developing reliable predictive biomarkers of benefit from immunotherapy and in better defining which immune factors regulate response. Indeed, the immune system is a constantly changing entity which adapts and responds to a complex matrix of mediators including: tumor neoantigens, antigen presenting cells, co-stimulatory cell-surface proteins, proliferative and differentiating cytokines, extracellular matrix cell trafficking influences, and natural or tumor-induced inhibitors of inflammatory immune function [2]. While adaptive immunity in the form of memory T-cells is a key component in the ability of humans to successfully mount an immune response to a previously encountered infectious or malignant antigenic stimulus, it is abundantly clear that the proportions of antigen-specific T-cells fluctuate over time in tune with variations in antigen exposure. While investigators have demonstrated that clonal evolution of tumors can result in addition and occasional loss of gene mutations over time, gene mutations are generally thought to be a less plastic observation than the proportional composition of a patient's T-cell populations [3]. Thus, successful identification of reliable, validated, biomarkers of response to immunotherapy

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poses considerable challenges. Recently though, several molecular analysis platforms offer potential strategies to address these challenges with each demonstrating encouraging preliminary associations with immunotherapy clinical outcomes. Within this chapter, we will present an overview of the molecular pathology approaches with predictive potential for UCa patients treated with immunotherapy.

The Role of Programmed Death-Ligand 1 (Pd-L1) as a Biomarker of Treatment Response

PD-L1 Structure and Function

Programmed death-ligand 1 (PD-L1) is also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1). PD-L1 is expressed on the surface of tumor cells and induced on key regulators of the anti-tumor immune response including T-cells, natural killer (NK) cells, macrophages, dendritic cells, B-cells, and vascular endothelial cells [4]. Together with its natural ligand partner programmed cell death protein 1 (PD-1), PD-L1 functions to dampen T-cell mediated inflammatory responses. The crystal structure of PD-L1 is composed of two extracellular immunoglobulin (Ig) domains joined by a short linker, an N-terminal variable domain and a C-terminal constant domain. PD-L1 binds to PD-1 through its extracellular N-terminal Ig variable domain via 18 hydrogen bonds in a manner similar to variable domains found on antibodies and T-cell receptors. The conformation of the two terminal portions of PD-L1 is flexible and changes depending on whether PD-L1 is complexed with PD-1 or is in its free uncomplexed state (Fig. 10.1).

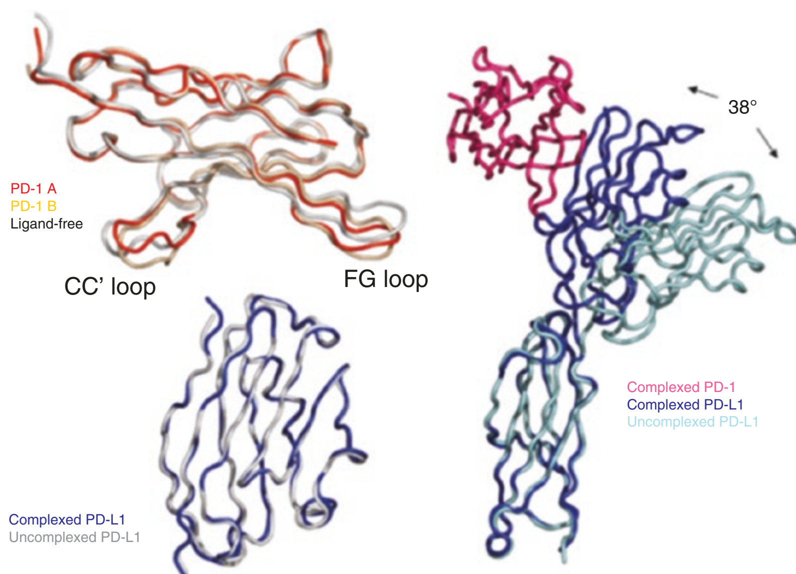

Fig. 10.1 Crystal structures of PD-L1 in complexed and uncomplexed states with PD-1 [5]. Copyright 2008 National Academy of Sciences

Within the structure of the PD-L1 variable domain, the residues Ala-121, Asp-122, Tyr-123, and Lys-124 on the G strand appear to be critical in binding to PD-1 [5].

PD-L1 and PD-1 Immunohistochemistry Observations from Non-urothelial Malignancies

The natural role of the PD-L1 and PD-1 pathway to prevent excessive inflammation and subsequent tissue necrosis is a necessary and ordinarily beneficial function. However, in the case of upregulation of PD-L1 on tumor surfaces or within the tumor microenvironment, including immune cells, the inhibitory effects on the antineoplastic immune response provide an escape mechanism for the tumor to continue to grow, invade, and metastasize. The pro-tumor effects of the PD-L1 and PD-1 pathway make them attractive targets for drug development. Indeed, multiple PD-L1 and PD-1 inhibiting monoclonal antibodies have demonstrated clinically relevant tumor response rates across a wide range of malignancies including: melanoma, non-small cell lung cancer, renal cell carcinoma, head and neck squamous cell carcinoma, adrenocortical carcinoma, Hodgkin's lymphoma, and urothelial carcinoma [1, 6–19].

In an effort to identify patients most likely to benefit from therapy, immunohistochemical expression of PD-L1 and PD-1 and relationship to immunotherapy response rates have been investigated in most of the studies. Based on early findings, expression of PD-L1 on tumor or inflammatory cells or expression of PD-1 on inflammatory cells appeared to associate with an increased response rate to

Table 10.1 PD-L1 as a biomarker of response to immunotherapy in non-urothelial malignancies

Author	Population	Agent	Target	PD-L1 Ab	Cutoff	PD-L1+ORR (%)	PD-L1- ORR (%)
Herbst [38]	mSolid tumors	Atezolizumab	PD-L1	SP142	5% IC	34	16
McDermott [14]	mRCC	Atezolizumab	PD-L1	SP142	1% IC	18	9
Horn [39]	mNSCLC	Atezolizumab	PD-L1	SP142	10% IC or TC	45	14
Daud [40]	mMel	Pembrolizumab	PD-1	22C3	1% TC	53	6
Garon [10]	mNSCLC	Pembrolizumab	PD-1	22C3	50% TC	45	15
Motzer [15]	mRCC	Nivolumab	PD-1	28-8	5% TC	31	18
Brahmer [9]	mNSCLC	Nivolumab	PD-1	28-8	5% TC	21	15
Callahan [41]	mMel	Nivolumab + Ipilimumab	PD-1/ CTLA-4	28-8	5% TC	40	47
Hammers [11]	mRCC	Nivolumab + Ipilimumab	PD-1/ CTLA-4	28-8	1% TC	50	55
Larkin [12]	mMel	Nivolumab + Ipilimumab	PD-1/ CTLA-4	28-8	5% TC	72	55
Grasso [42]	mMel	Nivolumab	PD-1	28-8	5% TC	44	17
Topalian [19]	mSolid tumors	Nivolumab	PD-1	5H1	5% TC	36	0

monotherapy, as summarized in Table 10.1. When combination immunotherapy regimens are utilized, however, the data is more variable. This may reflect a combination of factors related to both the immune system repertoire and development of a diverse array of antibodies to assess PD-L1 and PD-1 expression. First, several different commercial PD-L1 IHC antibodies have been used within the trials to date. The operating characteristics of each antibody can vary significantly making comparisons between studies difficult (Fig. 10.2). Second, the definitions of a "positive" PD-L1 IHC stain are fine-tuned for each antibody, with differences in both in cut-off

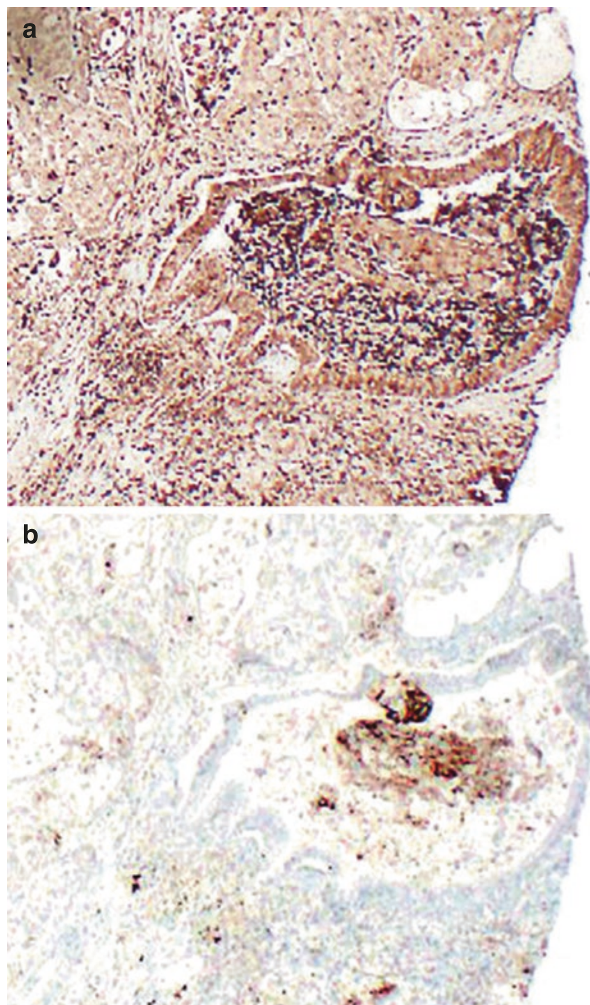

Fig. 10.2 Immunohistochemical stains using two different antibodies targeting PD-L1 on sequential sections of the same tumor sample. (**a**) This sample shows strong staining in the majority of tumor and infiltrate cells, although there appears to be a high background. (**b**) This antibody shows occasional staining of the tumor cells and more variability in the intensity of immune infiltrate staining

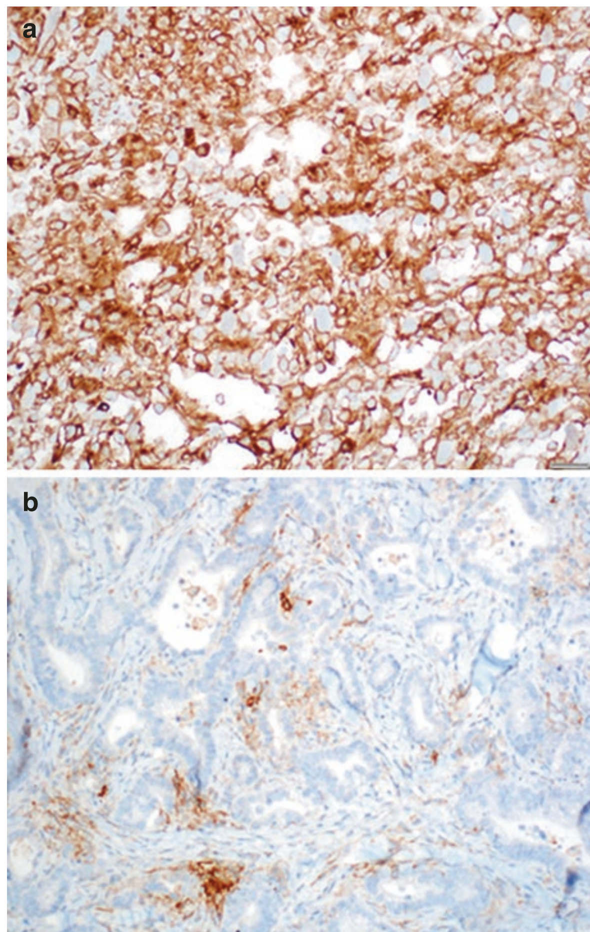

Fig. 10.3 Different elements of the cancer landscape show staining. (**a**) This high-magnification view of a urothelial carcinoma shows strong and diffuse PD-L1 immunostaining of the cancer cells. However, distinguishing staining of infiltrating lymphocytes in this setting is challenging. (**b**) This adenocarcinoma shows an absence of PD-L1 staining using the same antibody, but in this case strong PD-L1 expression is present in infiltrating lymphocytes

for percent staining and in cell type that is stained (immune cell (IC) vs tumor cell (TC); Fig. 10.3). Third, the location of the tumor-associated lymphocytes has not been factored into most analyses, which is problematic when lymphocytes are not uniformly distributed. For example, some tumors show robust tumor infiltrating lymphocytes (TILs), whereas other may lack associated lymphocytes or enrich for them only at the tumor-stroma interface. Thus, sampling bias introduced by the tumor sample location and number of biopsy cores could produce an inaccurate representation of PD-L1 status based on number of tumor cells sampled, location of TILs, and heterogeneity of PD-L1 and PD-1 expression. Fourth, due to the plasticity of the immune system, PD-L1 expression is likely to vary over time in tune with the ebb and flow of antigen presentation and subsequent inflammatory infiltrate within the tumor and its microenvironment. Therefore, an older archived tumor biopsy which demonstrates no PD-L1 expression cannot be considered a reliable indicator of current PD-L1 status. The exact time window within which a tumor biopsy is considered an accurate representation of PD-L1 status has not established. Lastly, in

almost all studies responses were observed in a subset of PD-L1 negative patients. In a prior chemotherapy driven era, these lower response rates were usually discarded as clinically irrelevant. However, the observation that responses to PD-L1/PD-1 therapy, when they occur, tend to be durable has been a common theme. In instances in which alternatives are not available, pre-selecting patients based on PD-L1 or PD-1 status has not been recommended. However, as PD-L1 and PD-1 based immunotherapy moves to possible first-line options for some patients, a more reliable method to determine likely responders will become important, given the cost and potential toxicity of immunotherapy regimens.

Clinical Experiences with PD-L1 Immunohistochemistry (IHC) in Urothelial Carcinoma

Encouraged by promising initial results in non-urothelial malignancies, rapid and intense PD-L1/PD-1 drug development efforts in UCa have followed. To date, three antibodies targeting PD-L1 (atezolizumab, durvalumab, avelumab) and two antibodies targeting PD-1 (pembrolizumab, nivolumab) have reported consistent tumor responses with very tolerable safety profiles in metastatic UCa patients. The predictive nature of PD-L1 IHC staining has shown mixed results in these studies summarized in Table 10.2.

In the initial data for atezolizumab from 67 metastatic UCa patients treated in a phase 1 trial, patients with PD-L1 IHC 2 or 3 intensity in ICs utilizing the SP142 antibody demonstrated a 43% objective response rate (ORR) compared to only 11% for IHC 0 or 1 patients [20]. In the follow-up phase 2 IMvigor210 trial in 310 metastatic post-platinum UCa patients treated in study cohort 2, utilizing the same

Table 10.2 PD-L1 as a biomarker of response to immunotherapy in urothelial carcinoma

Author	Population	Agent	Target	PD-L1 Ab	Cutoff	PD-L1+ORR (%)	PD-L1- ORR (%)
Powles [20]	2L mUC	Atezolizumab	PD-L1	SP142	5% IC	43	11
Rosenberg [1]	2L mUC	Atezolizumab	PD-L1	SP142	5% IC	26	9
Balar [8]	1L Cis-ineligible mUC	Atezolizumab	PD-L1	SP142	5% IC	24	24
Massard [13]	2L mUC	Durvalumab	PD-L1	SP263	25% IC or TC	46	0
Apolo [7]	2L mUC	Avelumab	PD-L1	73-10	5% TC	50	4
Plimack [16]	2L mUC	Pembrolizumab	PD-1	22C3	1% IC or TC	29	0
Sharma [17]	2L mUC	Nivolumab	PD-1	28-8	1% TC	24	26

antibody, patients with PD-L1 IC 2/3 intensity had an ORR of 26% compared to only 9% for PD-L1 IC 0/1 intensity [1]. In addition, after 17.5 months median follow-up, the median overall survival was 11.9 months in the PD-L1 IC 2/3 patients compared to only 6.7 months in the IC 0/1 group [21]. Similarly, in a phase I trial of durvalumab in 61 metastatic UCa patients, an ORR of 46% in PD-L1+ 0% in PD-L1- tumors was observed utilizing the SP263 antibody and assessing IHC intensity in either ICs or TCs [13]. In the case of avelumab in 44 metastatic UCa patients using the 73-10 antibody and a PD-L1+ definition of >5% TC staining, PD-L1+ patients have an ORR of 50% compared to only 4% in PD-L1- patients [7]. Lastly, in 33 metastatic UCa patients treated with pembrolizumab, an ORR of 29% was observed in PD-L1+ patients compared to 0% in PD-L1- patients with IHC evaluation of both ICs and TCs by the 22C3 antibody [16].

In contrast, no association was observed between PD-L1+ IHC status and ORR in 78 metastatic UCa patients treated with nivolumab as assessed with the 28-8 antibody using a 1% TC threshold (PD-L1+ 24%, PD-L1- 26%) [17]. Likewise, when atezolizumab was studied in 119 platinum-ineligible first-line metastatic UCa patients, no significant differences in ORR were observed between IC 2/3 (28%) and IC 0/1 (22%) patients [8]. These results were seen utilizing the same SP142 antibody as was used in the initial phase I and IMvigor210 trials.

Despite the finding that a subset of UCa studies suggest a trend between increased intensity of PD-L1 IHC staining and ORR, these findings do not show adequate sensitivity or specificity to be reliably used to determine patient response to immunotherapy at this time. Potential factors contributing to differences in predictability include the unique operating characteristics of the antibodies used, currently undefined tumor immunology differences between the chemo-naïve and post-platinum metastatic UCa patient populations, patient germline differences that alter immune cell responsiveness, and the local tumor-immune cell environment. These and other hypotheses require further investigation.

Impact of Molecular Subtype on Immunotherapy Response

In 2014, initial data from The Cancer Genome Atlas (TCGA) project analysis of urothelial carcinoma was first published [22]. For the first time, these results presented a comprehensive roadmap to the molecular biology underpinnings of muscle-invasive UCa. In addition to demonstrating alterations in genes with therapeutic drugs in development in 69% of patients, TCGA identified and classified patients into the two molecularly defined intrinsic basal and luminal subtypes based on several similarities to the corresponding subtype terminology utilized in breast cancer classifications. The UCa patients were subdivided further into four subtypes (Luminal Cluster 1, Luminal Cluster 2, Basal Cluster 3, Basal Cluster 4). In parallel to the TCGA effort other investigators developed similar molecular subtype classification systems based on next generation sequencing analysis of muscle-invasive UCa specimens. While the total number of subtypes varies

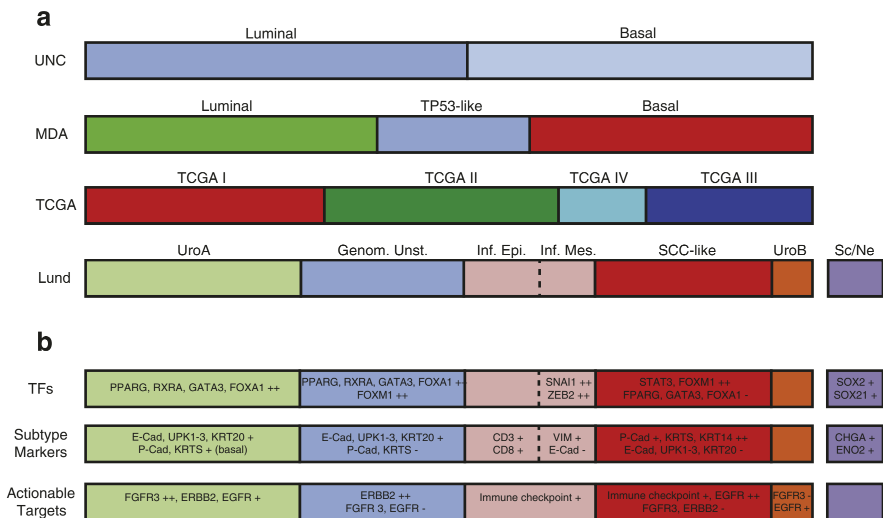

Fig. 10.4 (**a**) Proposed molecular subtypes of urothelial carcinoma; (**b**) Proposed subtypes of urothelial carcinoma based on marker expression

between as few as 2 to as many as 6 subtypes (Fig. 10.4), all are in agreement of the existence luminal and basal-like subtypes which possess fundamentally differing molecular biology signatures [23–27]. Luminal tumors are characterized by a papillary phenotype with activating FGFR3 mutations with enrichment for ER, GATA3, and PPARG. In contrast, basal tumors are typically more aggressive tumors which demonstrate increased expression of STAT3, TP63, KRT5/6A, CD44, NKkB, c-Myc, and HIF signaling. These fundamentally different molecular subtypes have been associated with responsiveness to cisplatin-based chemotherapy in initial retrospective investigations. A tumor infiltrative inflammatory phenotype has also been proposed for a subset of UCa patients and this may be relevant in the context of immunotherapy approaches.

Based on these observations, response to atezolizumab therapy according to TCGA-defined molecular subtypes was examined within the post-platinum metastatic UCa patients treated in cohort 2 of the IMvigor210 trial. Amongst the 195 patients who had tissue sufficient for TCGA classification, ORR varied significantly according to TCGA subtypes with 34% of luminal cluster 2 patients responding compared to only 10% of luminal cluster 1 ($p = 0.0017$) and 16% in basal cluster 3 and 20% in basal cluster 4 [1]. Results in the chemo-naïve cohort 1 patients from IMvigor210 and from trials of other UCa immunotherapy trials are eagerly anticipated to determine if enrichment of specific molecular subtypes can confer improvements in clinical outcomes to immunotherapy in UCa patients. At a minimum, the provocative observation of the impact of molecular subtype on atezolizumab response rate warrants analysis of molecular subtypes on future UCa immunotherapy trials.

Role of Neoantigens and Mutational Burden

The role of the immune system in preventing tumor initiation, growth, and metastases is highlighted by the long-standing observation of increased rates of cancer (squamous cancers, lymphomas, Kaposi's sarcoma) in immunosuppressed organ transplant or HIV patients. Conceptually, in order for the anti-neoplastic effect of the immune system to occur, either a disproportionate expression of non-mutated proteins concentrated in specific organ tissues or peptide alterations of mutated proteins presented on the cell surface in the context of major histocompatibility complexes (MHCs) must occur. The latter mutated proteins which do not exist in any normal tissues are termed neoantigens [28]. In order for a neoantigen to elicit an anti-neoplastic effect, the mutated protein must be processed and its resulting peptide sequence presented by MHCs. In addition, the peptide-MHC complex must be co-located in a T-cell rich microenvironment. Unless both of these conditions are met, an anti-neoplastic immune effect is unlikely. With the advent of modern next generation sequencing platforms, investigators have now catalogued the mutational profiles of most human malignancies. Shown in Fig. 10.5 are the "mutational burden" profiles across a wide spectrum of common human malignancies including UCa [29]. It is worth noting that tumors with the highest mutational burden (melanoma, lung, stomach, bladder) have all demonstrated responsiveness to modern immunotherapy approaches.

In UCa, investigators have begun to examine the impact of mutational burden as a biomarker for immunotherapy response. In the IMvigor210 atezolizumab trial, tumor mutational burden (mutations per megabase) was assessed by next generation sequencing in 150 post-platinum metastatic UCa patients (cohort 2) utilizing the 315-gene DNA-based FoundationOne® panel. Patients demonstrating tumor responses to atezolizumab therapy had significantly higher

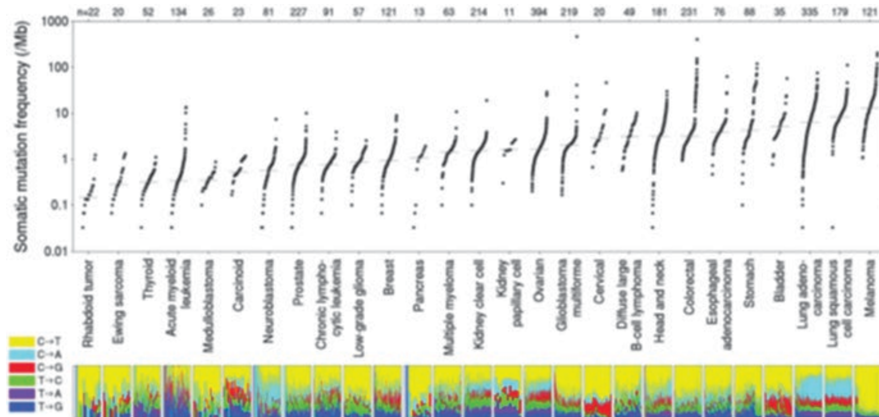

Fig. 10.5 Mutational burden of human cancers. Reprinted by permission from [29]. Copyright 2013 Macmillan Publishers Ltd

mutational burdens (12.4 vs. 6.4 mutations/megabase $p < 0.0001$) compared to non-responders [1]. This observation held true across all TCGA subtypes or PD-L1 IHC staining groups. In addition, the association between high mutational burden and improved response rates was also observed amongst chemo-naïve cohort 1 patients [30]. Furthermore, increased mutational burden has also been associated with improved recurrence-free survival in patients undergoing cystectomy for muscle-invasive UCa [31]. These additional reports suggest the potential utility of mutational burden as an immunotherapy biomarker that is independent of chemotherapy exposure history. Additional investigations are required to determine if any common neoantigens are conserved across significant proportions of patients and to assess the surrogacy of mutational burden estimates from focused tumor gene panels compared to full genome assessments. Nonetheless, mutational burden shows much promise as a predictor of benefit from immunotherapy for UCa patients.

T-cell Receptor Diversity Effects on Immunotherapy Response

Traditionally, an anti-neoplastic immune response was conceptualized by both innate and adaptive mechanisms. The innate immune response was thought to occur upon initial encounter with antigenic stimuli and was not dependent on prior antigen exposure. In contrast, the adaptive immune response relied upon a complex interplay between antigen presenting cells and various lymphocyte cell subsets to impart anti-neoplastic cytotoxic effects and confer long-term antigen-specific memory to prevent tumor growth and recurrence. While this dichotomous view of the innate and adaptive responses formed the cornerstone of tumor immunology research for several decades, it has been revised to a more contemporary mechanistic understanding in which the innate and adaptive immune responses do not occur in isolation but rather simultaneously and with complementary roles. Modern descriptions of the anti-tumor immune response typically employ a three-phase view composed of elimination, equilibrium, and escape phases [32]. In each of these phases, T-cells (CD8, CD4, Tregs) play a critical role in the immune system anti-tumor effects.

T-cell maturation is characterized by antigen-specific recognition via their cell membrane associated T-cell receptors (TCRs). TCRs are extremely variable proteins composed of an α- and a β−chain. A diverse spectrum of TCRs results from spontaneous recombination of the variable (V), diversity (D), and joining (J) exons. Additional splice events further increase TCR variations. Ultimately these VDJ rearrangements form the complementarity determining region 3 (CDR3) which is responsible for clonal antigen recognition [33]. High throughput next generation sequencing now permits analysis and identification of the each individual TCR. Thus, comprehensive assessments of TCR diversity are now possible. TCR diversity is typically reported as the Simpson's diversity index (DI) which ranges between 0

and 1 and represents the probability that any two randomly chosen TCRs will have different clonotypes (distinct CDR3s for the TCR α- or β-chain) [34].

In initial UCa investigations of the impact of TCR diversity on clinical outcomes, TCR diversity appears to have a prognostic role. In a retrospective analysis of muscle-invasive UCa tumor tissues from 38 patients undergoing cystectomy in the absence of neoadjuvant chemotherapy, patients with low TCR diversity demonstrated significantly longer recurrence-free survival compared to those with high TCR diversity ($p = 0.018$) (Fig. 10.6) [31]. Additionally, amongst the 29 metastatic post-platinum UCa patients in cohort 2 of the IMVigor210 trial of atezolizumab, patients with lower baseline TCR diversity assessed on the ImmunoSEQ® platform had significantly improved progression-free survival ($p = 0.0514$) and overall survival ($p = 0.0116$) [35]. The implication of these findings is that patients with low TCR diversity are those in which a successful antigen-specific adaptive immune response has occurred which, thereby, expands the antigen-specific proportion of TCRs, thus reducing the overall TCR diversity and imparting a beneficial antineoplastic effect. This association between improved UCa clinical outcomes and low TCR diversity is consistent with observations in other malignancies [36, 37]. Thus, UCa patients who demonstrate low TCR diversity in the setting of a PD-L1 rich TIL infiltrate may be those patients most likely to experience benefit from PD-1/PD-L1 immunotherapy approaches. Prospective studies of the predictive accuracy of TCR diversity as an immunotherapy biomarker in UCa are clearly warranted.

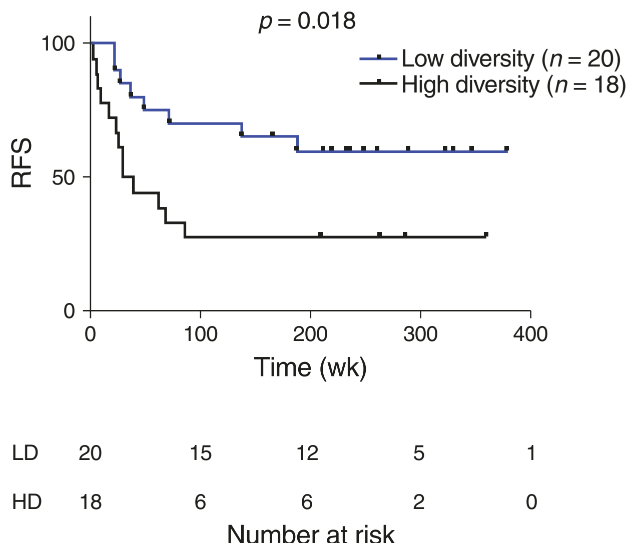

Fig. 10.6 Relapse-free survival after cystectomy according to T-cell receptor diversity in patients with muscle-invasive urothelial carcinoma of the bladder [31]. Reprinted with permission from European Urology. Copyright 2015 Elsevier

Conclusions

While UCa was one of the first malignancies to demonstrate clinically meaningful improvements in outcomes with the use of immunotherapy in the form of intravesical BCG, the recent advances observed with the use of PD-1/PD-L1 agents have sparked renewed enthusiasm for the clinical utility of additional immunotherapy approaches. Due to the inherent mechanistic differences between immunotherapy and traditional cytotoxic chemotherapy, an urgent need exists for improved pathology platforms that can identify reliable predictors of response and characterize mechanisms of resistance. PD-L1 immunohistochemistry, UCa molecular subtyping, neoantigen targeting, mutational burden quantification, and TCR diversity profiles each show encouraging early associations with responses to immunotherapy in UCa patients. To move each of these platforms forward to become a clinical decision-making biomarker utilized in day-to-day care of UCa patients, especially for immunotherapy treatment in the first-line setting, will require assay validation and confirmatory prospective trial investigations. In the next decade, it is likely that at least some of these promising molecular analysis platforms and others, yet to be discovered, will successfully complete the required validation steps to achieve this goal.

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Chapter 11
Emerging Molecular Approaches in the Analysis of Urine in Bladder Cancer Diagnosis

James P. Solomon, A. Karim Kader, and Donna E. Hansel

Introduction

A urine-based assay that could accurately screen for urothelial carcinoma (UCa) and/or reliably detect recurrent disease does not yet exist. Currently, the majority of newly diagnosed patients are identified by evaluation for gross or microscopic hematuria or workup of lower urinary tract symptoms. At this time, evaluation of the patient with clinical suspicion consists of cystoscopy, urine cytology, and CT urogram, as described in Chap. 8 [1]. In addition, a number of existing urine tests are commercially available, although have not found broad application due to limitations in sensitivity or specificity. In this chapter, we will review.

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Urine Cytology and Existing Commercially Available Urine Tests

Direct visualization of the urinary tract through cystoscopy and/or ureteroscopy, combined with histologic examination of biopsy material, is considered the gold standard for diagnosing urothelial carcinoma and precursor lesions, although it is less sensitive for detecting flat lesions than for detecting papillary lesions. Newer technologies such as narrow band imaging (NBI) and blue light cystoscopy continue to improve the sensitivity of direct visualization, but these do not alter the invasiveness and cost [2, 3].

Cystoscopy is often used in conjunction with urine cytology, which is performed by microscopic examination of exfoliated cells in voided urine or bladder washings. The benefits of cytologic evaluation of voided urine include its non-invasive nature, sensitivity to detect high-grade carcinoma, and ability to detect upper tract carcinoma. However, it is less sensitive for low-grade tumors and suffers from poor inter- and intraobserver agreement [4]. In recent years, standardization of the cytology classification of urine samples has been performed, with the publishing of the Paris classification system [5]. The goal of standardization is to enable more accurate diagnoses, reduce inter- and intraobserver discordance, and improve communication with clinicians. Nonetheless, the use of additional non-invasive testing could be beneficial to improve sensitivity, especially in screening for low-grade tumors.

For these reasons, a urine-based test that is non-invasive, inexpensive, rapid, and easy to use for both the initial diagnosis and the follow up of UCa has been the subject of intense study. It would seem that UCa would be the ideal disease for developing such a test, as it is in direct contact with a body fluid that is able to be easily and non-invasively obtained. However, to date, no reliable biomarker or genetic test has shown adequate sensitivity and specificity to replace, or even be routinely used in conjunction with, cystoscopy and cytology as the standard of care. However, several tests are commercially available, and there are many more that are currently under development that could be used to aid in the diagnosis of UCa. A detailed summary of the commercially available tests is presented in Chap. 8.

In a recent meta-analysis that examined 57 studies, the accuracy of NMP22, BTA, UroVysion, Immunocyt, and Cxbladder tests was evaluated using cystoscopy and histology as the reference standard. Sensitivities of the tests ranged from 57 to 82% while specificities ranged from 74 to 88%. In all cases, urinary biomarkers plus cytologic evaluation was more sensitive than biomarkers alone, but even with the best combination, still missed about 10% of cases. In addition, cytology plus biomarkers was not very sensitive for low-stage and low-grade tumors [6]. Overall, the findings suggest that the commercially available tests should be used in conjunction with cytology and cystoscopy to increase sensitivity, as currently, none appear to be an effective replacement for direct visualization by cystoscopy and pathologic examination of biopsy specimens and voided cytology. The results from this study suggest that new, improved urine biomarkers may be valuable in the diagnosis of UCa.

Clinically Promising Biomarkers for Urine-Based Testing

FGFR3 Mutations

FGFR3 mutations occur in approximately 50% of urothelial carcinomas, and while traditionally associated with low-grade papillary non-invasive lesions, they are also often seen in high-grade and invasive carcinoma. Traditionally, low-grade lesions are especially difficult to screen for, as the sensitivity and specificity for detecting these lesions by traditional urine cytology is low. Given the relative indolence of low-grade tumors, the clinical utility of a biomarker for this entity alone is controversial. A polymerase chain reaction (PCR) test was developed that can test for a few of the commonly seen mutations in *FGFR3* that are associated with low-grade urothelial neoplasms, allowing for easy detection in the urine [7]. This assay was prospectively tested in patients that had a history of low-grade non-invasive UCa, and it was found that analyzing the voided urine for *FGFR3* mutations had a sensitivity and specificity of 58% and 81%, respectively, for detecting recurrences [8]. Assessing for *FGFR3* mutations has also been demonstrated to be useful in detecting upper urothelial tract carcinomas [9]. In addition, in subsequent follow-up after treatment, a positive *FGFR3* mutation analysis appears predictive of recurrence [10].

Aurora Kinase A

Aurora A kinase is a serine/threonine kinase, encoded by the *AURKA* gene, which plays an important role in formation of the mitotic spindle. Aurora kinase A has been shown to be overexpressed in urothelial carcinoma and is associated with higher stage tumors and worse outcomes [11, 12]. A recent study examined *AURKA* mRNA expression in the urine from urothelial carcinoma patients and control patients without UCa. The mRNA levels, quantified by reverse-transcriptase PCR, were shown to be significantly higher in UCa, and sensitivity and specificity were found to be 83.6% and 65.2%, respectively. The *AURKA* assay was compared to cytology, and was demonstrated to be more accurate for low-grade tumors. Cytology, however, was better for picking up the high-grade tumors [13]. A second study used fluorescent in situ hybridization to examine *AURKA* gene amplification in exfoliated urothelial cells in urine, demonstrating a specificity of 96.6 and 87%, respectively, in a validation cohort [14]. Overall, assessment of genomic amplification or mRNA levels of *AURKA* appear to be promising ancillary tests for detection of UCa.

BLCA-1 and BLCA-4

BLCA-4 and BLCA-1 are proteins in the nuclear matrix, similar to NMP22. BLCA-1 is present in UCa, but is not present in adjacent uninvolved urothelium or in bladder tissue from normal patients [15]. In patients with UCa, BLCA-4

expression can be detected in both tumor and uninvolved urothelium, but is absent in the urothelium from normal patients [16]. Both proteins can also be detected in the urine and appear to be sensitive and specific biomarkers for the presence of UCa [15, 16].

Survivin

Encoded by the gene *BIRC5*, survivin is a member of the inhibitor of apoptosis family of proteins that regulates activation of caspases. It is present in most human tissues, but is normally absent in terminally differentiated cells. It has been demonstrated to be upregulated in many human cancers, and is therefore a promising biomarker and therapeutic target [17]. Survivin can be quantified in urine by either reverse transcriptase PCR or immunoassays. A recent meta-analysis examined survivin as a urine biomarker for UCa and demonstrated a sensitivity of 77.2% and a specificity of 91.8%. When compared to cytology, survivin was more sensitive, but not as specific [18]. In addition, a number of studies have demonstrated increased urine survivin levels to be associated with higher tumor grade and poor outcomes, including worse recurrence-free survival, cancer-specific survival, and overall survival [19, 20].

Clusterin

Clusterin is a heat shock protein that facilitates folding of secreted proteins in the Golgi. It also appears to play a role in tumorigenesis. Both serum and urine clusterin appear to be significantly higher in patients with bladder cancer than in controls. Protein levels of clusterin in the urine, as measured by ELISA, was shown to have a sensitivity and specificity of 87.1 and 96.7% respectively. In addition, higher clusterin mRNA expression, as measured by RT-PCR, was shown to be associated with tumor recurrence, higher stage, and worse overall survival [21]. It has also been suggested that using clusterin in conjunction with cytology improves sensitivity for UCa [22].

Cytokeratins

Cytokeratins are keratin-containing proteins that are important components of the cytoskeleton of epithelial cells. Different types of epithelium often have differing cytokeratin expression signatures, and the presence of neoplasia can often be detected by alteration in the cytokeratin expression. A few cytokeratins, including CK20, are useful in diagnosing urothelial neoplasia on surgical specimens [23, 24].

In addition, recent studies have demonstrated that certain cytokeratins in the urine may be associated with the presence of bladder cancer.

A fragment of cytokeratin 19, CYFRA 21-1, appears to be associated with UCa. In a meta-analysis that compared over 1200 UCa patients with 1200 non-bladder cancer (normal) patient controls, urine CYFRA21-1 was found to be 82% sensitive and 80% specific for the presence of UCa. Serum levels of CYFRA 21-1 can also be measured, which appears to be more specific, but less sensitive [25]. Another study examined patients with a history of UCa status post transurethral resection. Urine levels of CYFRA 21-1 were compared to cytology and ultrasound for detecting the presence of UCa, using cystoscopy as the gold standard of diagnosis. CYFRA21-1 levels alone were the most sensitive of the three screening methodologies, but still a few false-negative results occurred that were able to be identified by the other methodologies [26].

Telomerase

Telomerase adds a repeat sequence of DNA to the ends of chromosomes via reverse transcriptase activity. It functions to protect the ends of dividing chromosomes, and is therefore active in stem cells and cancer cells, but often lost in terminally differentiated cells. The presence of telomerase therefore is a potential tumor marker. The structural components of telomerase include an RNA component that serves as a template, a telomerase reverse transcriptase (TERT), and other associated proteins. A number of assays can be used to detect telomerase. In the telomeric repeat amplification protocol (TRAP), telomerase activity is measured by allowing telomerase to synthesize elongation products, which are then PCR amplified to allow for accurate quantification. Alternatively the RNA component or the mRNA for TERT can be quantified by reverse transcriptase PCR [27]. All three methods can be used to detect telomerase in urothelial cells in voided urine. Measuring mRNA for TERT appears to be the most sensitive, and also may be most sensitive for high-grade UCa [27, 28]. However, other studies have also demonstrated reasonable sensitivity and specificity for distinguishing UCa patients from normal controls using the TRAP assay [29].

Hyaluronic Acid

Hyaluronic acid is a nonsulfated glycosaminoglycan that is an important component of the extracellular matrix. It is synthesized by three synthetases (HAS1, HAS2, and HAS3) and interaction with HA receptors, such as CD44 can promote tumorigenesis and the epithelial to mesenchymal transition [30]. Additionally, hyaluronic acid is cleaved by the hyaluronidase HYAL-1, and the fragments of hyaluronic acid appear to promote angiogenesis of tumors [31].

The combination of hyaluronic acid and hyaluronidase, measured in an ELISA-like test called the HA-HAase test, has been demonstrated to be significantly more sensitive than the Immunocyt test and cytology, as tested by McNemar's test for paired sensitivities, while the specificity of all three tests was similar [32]. The sensitivity appears to be greater for high-grade tumors, with a recent study showing a 92% sensitivity for high-grade UCa [33].

Other methods can also be used to measure hyaluronic acid and associated synthetases or hyaluronidases. In a recent study, quantitative PCR and immunohistochemistry was used to demonstrate that expression of HAS2 and HYAL-1 could detect UCa with a sensitivity of 85.4% and a specificity of 79.5%. In addition, in a multivariate analysis, HYAL-1 and HAS1 expression correlated with metastasis and HYAL-1 expression correlated with disease-specific survival [34]. Finally, hyaluronidase activity can be measured by zymography. In this process, the urine supernatant is examined by electrophoresis in a polyacrylamide gel that contains hyaluronic acid. The activity of hyaluronase in the urine supernatant can then be visualized after staining with Alcian blue as a band where the hyaluronic acid has been degraded. Measuring hyaluronidase activity appears to be 89% sensitive and 90.5% specific for UCa [35].

Epigenetic Urinary Markers

Compared to many other cancers, UCa appears to harbor an increased number of genetic alterations that affect epigenetic modification, including chromatin remodeling, histone modification, and promoter methylation [36]. Methylation of CpG-rich regions in promoters regulates gene products, and aberrations in promoter methylation in particular seem to play an important role in UCa tumorigenesis. Some investigators have therefore been examining detection of aberrant promoter methylation as a possible urine biomarker for UCa. Quantitation of promoter methylation can be assessed using methylation-specific PCR, which uses two primer pairs to compare the methylated and non-methylated genes [37].

One of the most widely studied methylated genes is retinoic acid receptor beta, *RARβ*, which appears to have a sensitivity and specificity of 65% and 89.7%, respectively, when used alone [35]. However, examining *RARβ* in conjunction with methylation of other genes, such as *APC, BIRC5, DAPK, CDKN2A,* and *CDH1*, greatly improves the sensitivity of the screening test to a level at least as sensitive as cytology, while also enabling accurate detection of carcinoma in situ [38, 39]. Another study examined methylation of *BCL2, TERT,* and *DAPK* in a series of non-muscle invasive bladder cancers and compared them to normal controls. The findings demonstrated that the number of methylated genes correlated with tumor grade, and that methylated BCL2 and hTERT correlated with the presence of tumor [40].

Other studies have taken a more high-throughput approach to identification of potential methylated promoter region biomarkers. One group examined 1303 CpG sites, comparing tumor samples from 91 UCa patients, corresponding uninvolved

urothelium from the same patients, and 12 normal controls. They found that in tumor samples, there were 158 hypermethylated sites and 356 hypomethylated sites, and that a panel of 12 markers could reliably distinguish the tumor and normal samples. In addition, they demonstrated that identification of these markers in the urine could accurately identify the UCa patients [41].

Overall, the examination of methylation levels of various candidate genes is a current subject of intense study. While the genes examined in many studies vary, a common conclusion is that there are significant differences between methylation of CpG islands in gene promoters in urine from urothelial carcinoma patients and that of uninvolved patients. Sensitivities and specificities vary among the studies, and no panel has currently declared itself as superior over others and there is no current consensus for the genes that are best able to discriminate [42–48]. Nonetheless, the use of methylation assays appears to be a promising biomarker for the accurate detection of UCa.

Other mRNA Expression Panels

Although the CxBladder test is commercially available and one of the most widely used panels of mRNA for detecting UCa, a number of other panels of genes have identified that have adequate sensitivity and specificity for bladder cancer. One such study examined 44 different mRNA transcripts in urine samples from 89 patients with active UCa and compared them to 107 patients with a history of urothelial carcinoma but no current active disease. Using a multivariate analysis, the authors were able to identify a panel of 18 genes that was 85% sensitive and 88% specific for bladder carcinoma [49]. Another group examined 45 candidate genes and was able to identify a four gene expression signature that was associated with UCa. Further evaluation of those genes narrowed them down to two genes—*IGF2* and *MAGEA3*—that were able to predict urothelial carcinoma with a sensitivity of 81.5% and a specificity of 91.3% [50]. Overall, as additional gene panels are examined and studied, more mRNA based urine assays will likely be available.

Microsatellite Analysis

A few studies have shown that microsatellite analysis has a high sensitivity for early detection of bladder cancer. Urine can be sedimented, DNA extracted from urothelial epithelial cells, and microsatellite sequences examined [51]. PCR amplification using fluorescent probes is then performed, and capillary electrophoresis is used to separate and detect the amplification products. The results from the urine can then be compared to the patient's white blood cells. Some of the most widely used sequences for examining microsatellites are on chromosome 9, which is a frequent early alteration in urothelial carcinoma. Using this method, one study found that the

presence of at least one microsatellite alteration was found to have a sensitivity of 80.8% and a specificity of 85.1% [52]. Other studies have demonstrated microsatellite analysis to be more sensitive and specific than cytology and the BTA test and more sensitive than FISH [51, 53, 54]. In addition, it also seems to be effective for detecting upper urinary tract tumors [55].

microRNA

MicroRNAs are short segments of non-coding RNA that play an important post-transcriptional regulatory role in gene expression. MicroRNAs are dysregulated in many human cancers, including bladder cancer, and therefore their detection in urine could prove to be a valuable biomarker. MicroRNA can be found both in urine sediment and also as cell-free microRNA in urine supernatant. A number of studies have analyzed different panels of microRNA. One study demonstrated that two microRNAs, miR-125b and miR-126 showed a 10.42- and 2.70-fold increase, respectively, in urine from bladder cancer patients versus urine from age-matched healthy controls. A testing algorithm using these microRNAs in a validation cohort was able to detect urothelial carcinoma with 100% specificity and a sensitivity of 80% [56]. In another recent study, 15 microRNAs were quantified in 68 patients with bladder cancer and 53 age-matched controls. Differences in expression were seen in ten of the microRNAs, and by using a few in combination, the authors were able to detect bladder cancer with a high sensitivity [57].

Another study found microRNA signatures that were able to identify UCa—a two miRNA signature with an AUC of 0.83 and a six microRNA signature with an AUC of 0.92 [58]. One study even found that two microRNAs, miR-96 and miR-183, correlated with tumor grade and stage [59]. Finally, microRNA can also be epigenetically modified, and aberrant epigenetic alteration may also be a marker of UCa. One study identified a set of four microRNAs for which examining their methylation in urine could identify UCa with an area under the receiver operating characteristic curve of 0.916 [60]. Overall, even though there is no consensus on the best panel of microRNAs to assess, the analysis of microRNA in urine appears to be a viable strategy for detection of UCa.

Long Non-Coding RNA Assays

Long non-coding RNAs (lncRNAs) are long RNA transcripts greater than 200 nucleotides in length that do not code for any proteins. The lncRNA urothelial cancer-associated 1 (UCA1) is associated with the presence of UCa and also is associated with cisplatin chemotherapy resistance by activation of Wnt signaling [61]. A recent study showed that the lncRNA UCA1 could be directly detected in urine using a nanoparticle based hybridization assay that was 92.1% sensitive and

93.3% specific for UCa [62]. It is important to note that lncRNA, mRNA, microRNA, and proteins may all be present in exosomes, 30–150 nm membrane-bound secreted vesicles that are present in biological fluids. Exosomes can be isolated from the urine by ultracentrifugation for further analysis and can be an effective method of concentrating these analytes [63]. Overall, lncRNAs are novel promising biomarkers that deserve further study.

Protein Biomarkers by Mass Spectrometry

Mass spectrometry has been used to identify polypeptides in the urine that are specific for UCa that could be used to detect the presence of the disease. One recent study used capillary-electrophoresis-coupled mass spectrometry to compare urine protein signatures from 46 UCa patients and 33 normal controls. They were able to identify a panel of 22 polypeptides specific for UCa. When they used this panel on a prospective masked cohort, they were able to distinguish patients with UCa from normal volunteers with 100% accuracy and patients with UCa from patients with other urologic conditions with 86% accuracy [64]. One of the peptides that was identified in the panel was fibrinopeptide A, a known biomarker in ovarian and gastric cancers [64]. A second study also evaluated a panel of polypeptide biomarkers prospectively in 130 patients and showed a sensitivity of 81% and specificity of 57%. In addition, this second panel showed that it could be used to estimate the probability of muscle invasion [65]. Other studies, however, have demonstrated a much higher area under the receiver operating characteristic curve by analyzing panels of metabolites in urine [66]. Overall, similarly to some of the other biomarkers discussed above, a number of studies have demonstrated the functional utility of using mass spectrometry to evaluate panels of proteomic or metabolomic biomarkers, but consensus for the best panels to evaluate and consensus testing algorithm has not been developed.

Personalized Assays

Follow-up of patients with a history of UCa is a potential area where urine-based tests would be especially useful. When using cytology alone, it can sometimes be difficult to distinguish recurrent UCa from atypia that is caused by treatment changes, inflammation, or other sources. In addition, the differences in many of the urine biomarkers are often less able to detect residual or recurrent UCa. However, if certain attributes of a patient's tumor are known, a personalized assay can be developed to follow a patient. For example, if a tumor is demonstrated to have a specific *FGFR3* mutation, performing *FGFR3* mutation analysis at follow-up to look for that specific mutation has been demonstrated to be predictive of recurrence, both at the time of the assay and during subsequent follow-up [10].

In tumors that have mutated TERT promoters, these mutations can also be used to follow for recurrence. In a recent study, the same TERT mutations that were seen at diagnosis were present in urines of 7 of 8 patients that recurred, but were not seen in the six patients that did not recur. TERT promoter mutations are some of the most common mutations seen in non-invasive UCa and therefore could be useful for screening for recurrence [67]. Finally, some tumors may be negative for both FGFR3 and TERT mutations. A recent study was able to develop personalized assays by identifying other somatic variants in tumor DNA. By examining the serum or urine for these somatic variants, tumor DNA could be identified before definitive disease progression was seen by other testing modalities. Personalized assays may therefore be useful for monitoring disease recurrence or progression [68].

Conclusion

Many of the currently available urine-based tests for UCa show limited sensitivity and specificity benefit over cytology and cystoscopy for screening or routine follow-up. In certain situations, such as for monitoring for low-grade UCa or for occasions when cytology or cystoscopy are equivocal, certain tests have proven beneficial and their results affect diagnosis and treatment decisions. UCa is a very heterogeneous disease, with a high rate of somatic mutation and alterations in a wide variety of biological pathways affecting tumorigenesis. This may be one of the reasons an effective urine biomarker has not emerged. However, many exciting assays are currently in development that hold promise for an effective screening and/or monitoring urine test for UCa.

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Chapter 12
Stromal Contributions to Tumor Progression in Urothelial Carcinoma of the Bladder

Morgan Cowan, Daniel Miller, and Alexander S. Baras

Introduction

Urothelial carcinoma of the bladder is the fifth most common solid tumor and represents the most common and lethal malignancy of the urinary system [1]. Urothelial carcinoma is generally seen with increased age, with a median age at diagnosis of 70 years. Most patients have no apparent family history of urothelial cancers to suggest a heritable genetic component. Urothelial carcinoma is derived from the urothelium, the epithelial lining of the urinary tract, and as such begins as an in-situ, non-invasive process. In some cases, urothelial carcinoma develops invasive growth, beginning with extension into the stromal layer underlying and supporting the urothelium, the lamina propria. More aggressive tumors subsequently extend beyond the lamina propria and infiltrate the thick smooth muscle layer of the bladder wall, the muscularis propria. It is generally accepted that invasive growth, particularly into the muscularis propria, is a prerequisite for metastatic spread in urothelial carcinoma, presumably via involvement of lymphovascular channels of the bladder stroma.

Efforts to understand and characterize tumor progression in urothelial carcinoma, including both invasive growth and metastatic spread, have focused on the characteristics and molecular pedigree of urothelial carcinoma cells. Recent transcriptomic profiling of invasive urothelial carcinoma confirms the presence of distinct molecular subtypes [2, 3]. Most investigations have relied on gene expression to subtype urothelial carcinomas and these subtypes split primarily along the luminal/basal differentiation axis, similar to descriptions of breast carcinomas [4]. Overall, the genomic and transcriptomic landscape of urothelial carcinoma is

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vast and demonstrates a high degree of somatic mutational burden seen invasive urothelial carcinomas, third highest below non-small cell lung carcinoma and melanoma [5].

Although studies focused on urothelial carcinoma certainly have led to a better understanding of the molecular biology of tumorigenesis, it is also important to remember that this neoplastic process does not exist in a void. The physical environment around a tumor includes the supporting stroma with its myriad of components; including stromal fibroblasts and myofibroblasts, circulating immune cells, lymphovascular channels, and the supporting extracellular matrix. An evolving urothelial carcinoma has significant interactions with all of these different elements. While there certainly exist intrinsic tumor factors that increase the likelihood of tumor progression, it is becoming more and more important to appreciate the role of extrinsic factors in tumor progression. In this chapter we will review some of the relevant literature in these areas, primarily focusing on the stromal contribution to tumor progression, and discuss how this fits into our understanding of urothelial carcinoma.

Cellular Factors of the Bladder Stroma Associated with Tumor Progression

Stromal Cells

Stromal fibroblasts are one of the most basic cellular elements of the urothelial stroma. Fibroblasts produce collagen and other structural proteins to form the physical structure of the bladder. Such stromal cells, previously perceived to be mere bystanders in carcinoma growth, often show altered protein expression when in proximity to aggressive malignant tumors compared to stromal cells in normal tissues [6–8]. Such alterations imply a degree of stromal participation in tumor growth. Altered protein expression in the stromal cells provides biochemical signals to the surrounding epithelial cells, leading to subsequent modulation of epithelial cell behavior, and implying a pro-tumor role for the stroma. In one study, urothelial carcinoma cells exhibited a higher propensity for myoinvasion and increased growth kinetics when combined with fetal bladder stromal cells. Such in-vitro model systems of human urothelial carcinoma suggest the fetal stromal cells act to modulate tumor behavior and increase aggressiveness [9]. The stromal fibroblasts that are intimately associated with carcinoma cells are referred to as tumor-associated fibroblasts. A developing body of literature on tumor-associated fibroblasts indicates these cells act as a permissive factor in the development of an invasive carcinoma via interactions with other stromal cells [10]. Tumor-associated fibroblasts may further enhance tumor growth with modulatory effects on the host immune response against invasive carcinomas [11, 12].

Another stromal cell of interest is the myofibroblast, which possesses properties of both fibroblasts and smooth muscle, and in normal tissues supports epithelial cell proliferation required to maintain tissues [13]. Sub-urothelial myofibroblasts adhere to smooth muscle myocytes of the muscularis propria through specialized OB-cadherin-based adherens junctions not seen elsewhere in the bladder. The integrity of this myofibroblast–myocyte interaction could be affected by tumor-associated fibroblasts, creating a more permissive environment for tumor cell penetration [14, 15]. Furthermore, in bladder cancer, increased myofibroblasts in the lamina propria have been associated with muscle-invasive urothelial carcinomas as compared with non-muscle-invasive disease, suggesting a role for the myofibroblast in tumor invasion [16]. However, it is unclear as to whether this process is relevant to the initiation of an invasive carcinoma as opposed to a reactive phenomenon to an invasive carcinoma.

Cellular senescence, the permanent cessation of the capacity of a cell to divide, accumulates in cells with age, particularly fibroblasts. This phenomenon is of particular interest in urothelial carcinoma, given the increased average age of affected patients. Changes in fibroblasts inevitably affect the surrounding environment, and appear to decrease any inherent resistance to tumor cell growth. In advanced breast cancer, senescent stromal cells are central to the stromal changes supporting tumor cell seeding of bone in metastatic disease [17]. Mouse xenograft models of urothelial carcinoma have demonstrated that tumor progression can be potentiated by senescent stromal cells in conjunction with suppression of the host immune response to the tumor by recruitment of inhibitory myeloid cells [18]. The stroma–immune interaction further emphasizes that there are important interactions amongst the non-carcinoma cells of the tumor microenvironment that can affect tumor progression, particularly in the context of the modulation of the immune response.

Immune Cells

An increasingly recognized component of the stroma is the associated immune cells, which have complex and likely key interactions with a developing tumor. Although a detailed review of the tumor-immune interaction is beyond the scope of this chapter, it is evident that the degree of tumor infiltration by lymphocytes (TILs) is positively correlated with survival and prognosis in patients with urothelial carcinoma [19–22]. Autologous TIL lines have shown cytotoxic activity directed against tumor cells in a major histocompatibility complex type I (MHC-I) dependent manner [23]. MHC-I proteins display intracellularly derived peptide antigens on the cell surface to circulating cytotoxic T cells. Any recognition by the T cells of an MHC-I-associated peptide triggers T cell mediated destruction of the presenting cell. Not only should we consider stroma–tumor

interactions but also the role of potentially relevant microbiota of the bladder, which is certainly a relevant concept in urothelial carcinoma given the use of intravesical Bacillus Calmette-Guerin as treatment for non-muscle invasive urothelial carcinoma [24].

Mechanisms of immune evasion and tumoral immunogenicity are exciting areas of recent clinical successes in the treatment of bladder cancer [25, 26]. In order to better harness the benefits of targeted immune checkpoint inhibition, a more detailed characterization of the interactions between tumor cells and the immune system will become increasingly important. Additionally, the nature and composition of the immune cells that are interacting with the tumor cells will likely become more relevant for understanding both tumor progression and treatment-resistance in both muscle-invasive and non-muscle invasive forms of urothelial carcinoma of the bladder. It has been shown that increased tumor infiltration by host immune system lymphocytes tends to be associated with a better prognosis in muscle-invasive urothelial carcinoma of the bladder [20, 27, 28]. Already significant differences are emerging in the nature and composition of the immune cells interacting with invasive urothelial carcinoma across the tumor progression axis (pT1-pT4). Early invasive lesions not involving the muscularis propria (pT1) are met by a brisk inflammatory response including a gamut of important immune system cell types, including CD8+ (cytotoxic) T lymphocytes and CD20 positive B lymphocytes. More advanced and muscle-invasive urothelial carcinomas (pT3-pT4) display a sparser distribution of these same cell types. There are likely to be therapeutic implications to the nature and composition of the immune cells that are interacting with an evolving urothelial carcinoma. A recent study in muscle-invasive urothelial carcinoma showed that the overall degree of tumor infiltration by lymphocytes was not predictive of response to cisplatin-based neoadjuvant chemotherapy. Inspected more closely, the data show the right type of lymphocyte infiltrate could predict neo-adjuvant chemotherapy response in that the ratio of CD4 positive regulatory T cells to CD8 positive cytotoxic T-cells was predictive of response, but not simply the presence or absence of lymphocytes [29]. These findings would suggest that conventional chemotherapeutic treatment modalities are most optimally utilized in the context of an appropriately geared immune response. At present, the question of how to best optimize the patient immune response and how to increase this sensitivity remains unanswered.

We are on the precipice of a clearer and more mechanistic picture of how an evolving urothelial carcinoma interacts with the host immune system and what the implications are for tumor progression and therapeutic response. In more advanced disease, the increased overall mutational burden/rate observed in urothelial carcinoma should increase the likelihood of an immunogenic tumor cell derived antigen recognizable as foreign by the immune system, known as a neoantigen. Such a neoantigen would, theoretically, be more likely to stimulate a host immune response with resultant immune-mediated destruction of

tumor cells. This putative increase in immunogenicity may result in increased immune surveillance of the tumor, perhaps reflected in the increased TILs seen in some cases of advanced disease. Increased immune surveillance may come with very relevant therapeutic correlates, namely the induction of various mechanisms of immune evasion, such as immune checkpoint inhibition. Immune checkpoints are molecules and pathways which dampen the immune response. Normally, these checkpoints protect host tissues from destruction during immune activation by a non-self-antigen. This normally protective adaptation can be hijacked by tumors faced with an anti-tumor immune response in which tumors expressing particular immune checkpoint molecules may then evade the immune system (referred to as "immune escape"). One immune checkpoint pathway, the programmed cell death protein 1 (PD1)/programmed death ligand 1 (PD-L1) signaling axis, has emerged as a potent immunologic therapeutic target in bladder cancer [25, 26]. PD-L1 is part of an immune checkpoint pathway which helps normal cells avoid collateral damage during immune activation. PD-L1 recognition by activated immune cells abrogates immune system targeting of the PD-L1 expressing cells. When expressed by tumor cells or tumor assocated immune cells, PD-L1 enables tumor cells to avoid the immune system targeting despite potential immune system recognition. Recent clinical successes in the treatment of bladder cancer have exploited this and other mechanisms of immune evasion [25, 26]. In order to better harness the benefits of targeted immune checkpoint inhibition, a more detailed characterization of the interactions between tumor cells and the immune system will become increasingly important.

The role of the immune system as a disease modifying agent in bladder carcinoma is not limited to the immune cells themselves. There are also important interactions amongst the non-carcinoma cells (stromal and immune) that can affect tumor progression. Senescent stromal cells have shown the ability to recruit myeloid cells that inhibit anti-tumor CD8+ cytotoxic T-cells in the stroma and thereby promote tumor progression via immune inhibition [18]. The immune response to urothelial carcinoma also includes interaction with the urothelial microbiome. This microbial-tumor-immune interplay is certainly a relevant concept in urothelial carcinoma given the long-standing use of intravesical infusion of a mycobacterium, Bacillus Calmette-Guerin (BCG), as treatment for non-muscle invasive urothelial carcinoma [24]. Moreover, the nature of the immune response to BCG (such as Th1 vs Th2) is relevant to the understanding of progression of disease to muscle-invasive carcinoma despite conventional therapeutic modalities [30]. The host immune response to BCG organisms increases the exposure of adjacent tumor cells to an activated immune system, and presumably thereby increases immune clearance of tumor cells in addition to bacilli. Host immune surveillance and clearance of urothelial carcinoma may involve similar complex processes as those in BCG therapy response which could also have implications for tumor progression.

Lymphovascular Channels

Epithelial cells depend on stromal lymphovascular channels for blood and lymphatic supply. Similarly, epithelial-derived carcinomas require a robust vascular network both to support local growth and serve as the presumed transport vehicle to distant sites in metastatic disease. Tumor cells may promote vascular channel development both within and around the tumor by secretion of growth factors such as vascular endothelial growth factor receptor 3 (VEGFR-3) along with vascular endothelial growth factors C and D (VEGF-C/VEGF-D).

Newly formed vascular channels associated with malignant tissues exhibit remarkable morphologic differences from normal uninvolved vascular channels. Generally, the tumor-associated vascular channels are disorganized, tortuous, and excessively branched with significant luminal dilation compared to normal vascular structures of the body. The basement membrane and the external smooth muscle surface of tumor-associated vascular channels are incomplete or even absent. The endothelial cells lining the vascular lumens are abnormal in shape and organization, potentially due to reactive/regenerative changes, deviating from the pavement organization that is normally seen [31]. Perhaps unsurprisingly, these structural changes in vascular spaces decrease resistance to tumor cell penetration.

In invasive urothelial carcinoma, evidence of lymphovascular (LV) space involvement identified by routine pathologic assessment portends a worse prognosis [32]. Interestingly, increased amounts of lymphovascular channels associated with urothelial carcinoma is associated with worse prognosis in both invasive and non-invasive disease, independent of direct involvement of the lymphovascular channels by tumor [33, 34]. Additionally, current models of metastatic spread suggest that the ability to invade into lymphovascular spaces is not only a property of rare cells in advanced cancers, but is a continuous process seen across the spectrum of tumor progression.

The previous discussion has emphasized interactions amongst the various non-neoplastic elements of supporting stroma surrounding tumors (including tumor-associated stromal cells, immune cells, and also vasculature/lymphatics) are complex; a deeper and more detailed understanding of the entire tumor environment is critical to properly characterize all factors contributing to tumor progression.

Acellular Factors of the Bladder Stroma Associated with Tumor Progression

Stromal cells exist within a supporting physical framework, the extracellular matrix (ECM). The ECM is a complex and dynamic microenvironment consisting of both structural materials, termed the “core matrisome” which comprises approximately 200 glycoproteins, 40 collagen subunits, and 30 proteoglycans; as well as “matrisome-associated” molecules [24]. The latter includes hundreds of different

secreted factors that bind specific components within ECM such as matrix metalloproteinases (MMPs), transforming growth factor β (TGF- β), a hodgepodge of cytokines, growth factors, adhesion receptors and mucins, and myriad so-called ECM regulators. The secreted bound elements and supporting network of the ECM together create a spatially patterned milieu described as a "solid-phase organized assembly of ligands" that can be derived from both tumor cells and non-neoplastic cells, such stromal and immune cells [35]. Tissue engineering efforts have shown that ECM derived from healthy patients is a more tumor suppressive microenvironment compared to ECM from patients with colorectal tumors, suggesting the ECM surrounding tumors is fundamentally different in structure and/or function [36].

Additionally, altered protein expression by stromal cells changes the biophysical properties of the ECM [37, 38]. The ECM composition is also affected by stromal cell interactions with adjacent epithelial cells. Such dynamism of the ECM may affect the ability of probing carcinoma cells to develop and establish an invasive growth pattern. Interest remains in the search for genes associated with regulation of ECM remodeling and progression to muscle-invasive urothelial carcinoma. As previously mentioned, stromal cells secrete various paracrine and autocrine factors which include ECM-degrading proteins such as matrix metalloproteinases (MMPs). It has been proposed that the degradation of the ECM may favor tumor growth through several mechanisms. One mechanism is the release of growth factors when the ECM is destroyed, including epidermal growth factor (EGF) and TGF-β, which stimulate tumor cell growth. Secondly, ECM degradation exposes ligands recognized by tumor cell integrins, proteins involved in cellular adhesion to other molecules, and the integrin–ligand interaction may stimulate cancer cell growth via downstream RAS-induced pathways, such as the MAPK/ERK and the PI3K/Akt pathways [39, 40].

The current literature consistently implicates a particular extracellular proteoglycan, Decorin, as a modulator of tumor progression, which is worthy of further discussion. Decorin is a stromal leucine-rich proteoglycan that is secreted by fibroblasts. Decorin binds collagen type-I, serving as a sequestration anchor of several paracrine growth factors. The literature on Decorin thus far indicates that it can have both tumor suppressive and promoting properties, suggesting a complex and context-dependent interaction of stroma and tumor. As a stromal factor, Decorin can be potently anti-tumor. There are strong data to support a role for insulin-like growth factor receptor I (IGF-IR) in promoting invasive disease in bladder carcinoma. IGF-IR activation leads to activation of tumor molecules, such as Akt, p70S6K, and ERK, which are associated with increased tumor cell migration and invasion of deeper structures. Perhaps unsurprisingly then, IGF-IR is upregulated in muscle-invasive bladder carcinomas compared to both normal urothelial and non-invasive carcinoma [41]. Decorin binds IGF-1R and tempers its activation, subsequently decreasing tumor cell migration and invasion. By this mechanism, Decorin appears as a negative regulator of the invasive phenotype of bladder carcinoma [41]. Decreased Decorin expression in the tumor-associated stroma of bladder cancer, a phenomenon seen in other

tumor types, insinuates that stromal Decorin may indeed be anti-tumor [42]. Paradoxically, when expressed by the tumor cells themselves, Decorin promotes expression of angiogenesis related-genes and is seen in muscle-invasive bladder carcinoma [43]. The evolving story of Decorin emphasizes the complex and context-dependent nature of tumor–stromal interaction and the genes and proteins involved.

Another stromal factor to consider is the plasminogen activation system, a well-characterized proteolytic cascade important for invasion of many solid tumors. Urokinase-type plasminogen activator receptor (uPAR) is the receptor for urinary plasminogen activator (uPA), which cleaves precursor plasminogen to the active enzyme plasmin and thereby promotes subsequent degradation of extracellular matrix and basement membrane proteins. Both uPA and uPAR are primarily expressed in tumor-associated macrophages and myofibroblasts. uPA and uPAR expression by a number of solid tumors is a poor prognostic indicator. This system has not yet been well studied in the context of urothelial carcinoma. Recently, a study showed that increased uPAR expression in either the tumor cells or the associated stromal myofibroblasts and macrophages was significantly associated with muscle-invasive urothelial carcinomas, i.e. stage II and above [44]. Additionally, an association emerged between increased uPAR expression in the stromal myofibroblasts and decreased overall survival [44]. Here again the data support the key role stromal factors play in tumor progression and outcomes.

Summary and Conclusions

In contrast to the intrinsic characteristics of the tumor cells, the contribution of the stroma to tumor progression is generally an understudied area, which certainly is true in bladder cancer as well. Interestingly though, the key staging criteria in urothelial carcinoma of the bladder (along with other neoplasms) is based largely on the nature of the interaction of the tumor with the local structural environment, Fig. 12.1b. Additionally, although somewhat not directly related to tumor progression, the fact that non-invasive papillary urothelial carcinoma is able to develop presumably non-neoplastic underlying fibrovascular cores within its papillae to support this growth pattern is of particular relevance. In this review, we have attempted to highlight some of the cellular and acellular non-neoplastic stromal factors that appear to effect tumor progression.

More of these types of studies are needed to help elucidate the molecular mechanisms of the tumor–stroma interaction that influence tumor progression. An optimal future strategy may involve more integrated views and analyses of both intrinsic tumor factors and extrinsic stromal factors in the context of tumor progression and response to therapeutic approaches, as neither of these are completely independent processes.

Fig. 12.1 Overview of tumor staging and progression in urothelial carcinoma of the bladder. (**a**) Staging of bladder cancer according to the Tumor–Node–Metastasis (TNM) system is shown as adopted with permission from a recent review of bladder cancer by Knowles and Hurst in which the conventional tumor centric view is represented. Interestingly though, the key staging criteria in fact depend on how the tumor is interacting with the local structural environment from non-invasive, to superficial lamina propria invasive, and then to muscle-invasive carcinoma. (**b**) The tumor–stromal interaction across tumor progression in urothelial carcinoma of the bladder is shown, adopted with permission from a review by Mueller and Fusenig. Benign urothelium exhibits a normal axis of differentiation from basal to intermediate cells and then to surface umbrella cells. Normally, the urothelium is separated from the underlying stromal compartment of the lamina propria by a well-delineated basement membrane. This stromal compartment normally contains resting fibroblasts, mature blood vessels encircled by an uninterrupted basement membrane (capillary bed), and a few resident cells of the immune system (such as monocytes, lymphocytes, and granulocytes). In non-invasive carcinoma, the dysplastic epithelial cells are morphologically atypical and generally hyperplastic but the basement membrane remains intact, separating the epithelium from the underlying stromal compartment. Fibroblasts, however, become activated, and the number of macrophages increases. There is also some transient angiogenesis that occurs initially. In papillary carcinoma, the stromal compartment, including fibroblasts and capillaries, are "pulled" up by the developing carcinoma into an exophytic process supported by fibrovascular cores. Progression to an invasive carcinoma is associated with increased proliferation of epithelial cells (with increased mitotic activity) along with the development of an activated tumor stroma. Invasion by tumor cells begins through the degraded basement membrane, and blood vessels infiltrate the tumor tissue. Additionally, extracellular-matrix (ECM) components can be degraded by tumor, because of increased turnover and the lack of an intact basement membrane to restrict tumor cell growth. The number of inflammatory cells increases and fibroblasts differentiate into myofibroblasts, resulting in their expression of growth factors, matrix components, and degrading proteases. As this process continues, the invasive tumor cells gain access to the underlying muscularis of the bladder further potentiating the tumor–stromal interaction, including interactions with a gamut of cell types such as fibroblasts, myofibroblasts, smooth muscle cells, along with cells of the immune system

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Chapter 13
Modeling Bladder Cancer with Genetic Engineering: Fidelity of Human-to-Laboratory Models

Xue-Ru Wu

Why Do We Model?

A basic motivation to model cancer in mice is to produce a toolbox with which to evaluate new ideas in diagnostics, therapeutics, and prevention. Mouse models are generally considered superior to test tubes and cultured cells for these purposes because they provide a holistic physiological environment in which the test agents exhibit their biological effects and/or toxicities. Additionally, mouse anatomy, histological architecture, and genome are remarkably similar to the human counterparts, and information gleaned from the mouse models is by and large translatable to the bedside. Indeed, there are countless investigations that have utilized or even relied on mouse models to establish the basic parameters of new diagnostics and therapeutics that paved the way for human trials [1–5]. Some inroads have been made in the field of bladder cancer as well (Table 13.1), with more extensive use likely forthcoming with additional, human-relevant models in the pipeline (see later).

An equally important objective of mouse modeling is, through the development, characterization and extrapolation processes, to dissect the sequential steps of tumor formation and progression [6, 7]. Like tumors of other organ systems, bladder cancer develops through multiple stages and pathways, each involving the acquisition of genomic, genetic, and epigenetic changes [8–10]. While analysis of a human tumor specimen at any given time yields the totality of all the changes accumulated up to that point, mouse models provide a much more dynamic account of tumor

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Table 13.1 Utility of GEMMs in testing bladder cancer diagnostics, therapeutics and prevention

GEMM line	Phenotype	Test method/agent	Key outcome	References
Diagnostics				
UPII-SV40T (low-copy)[a]	CIS/high-grade, papillary tumors	Urine dipstick with micro-CT/3D imaging	Sensitive early tumor detection	[75]
UPII-SV40T (low-copy) NFkB-TRE-LacZ[b]	CIS/high-grade, papillary tumor	Micro-MRI and near infrared fluorescence Gd-Cy5.5 Live imaging	High lymphatic vessel density and activity	[76]
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	Near infrared fluorescence live imaging of Cy5.5-Tagged single chain VEGF	Activated VEGFR pathway in bladder cancer	[77]
Therapeutics				
UPII-HRAS*/*	Low-grade, papillary tumor	HDAC inhibitor belinostat	Significant inhibition of tumor via p21	[78]
UPII-SV40T/ UPII-HRAS*	High-grade, papillary tumor	mTOR, MAPK, STAT3 inhibitors	Synergism of the inhibitors on tumor growth	[68]
Floxed[c] p53/ PTEN Adeno-Cre (intravesical)	CIS/MIBC	Rapamycin (intravesical)	Blockage of CIS to MIBC progression	[51]
Floxed p53/ PTEN Adeno-Cre (intravesical)	CIS/MIBC	Cisplatin, gemcitabine, docetaxel (intravesical)	2-Agent combo involving gemcitabine most efficacious	[51]
Prevention				
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	Flavokawain A	Tumor inhibition and longer survival	[79]
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	CP-31398[d]	Inhibition of tumor formation and progression	[80]
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	Licofelone	Significant tumor inhibition	[81]
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	CP-31398 DFMO[e]	Tumor inhibition	[82]
UPII-SV40T (low-copy)	CIS/high-grade, papillary tumor	Rapamycin CP31398	Tumor inhibition	[83]
UPII-HRAS*	Low-grade, papillary tumor	Metformin	Urine concentration 240 fold that of blood; tumor inhibition	[84]

Notes:
[a]Low-copy, mouse line harboring low number of transgene copy
[b]NFkB-TRE-LacZ, NF-kB response elements driving a bacterial LacZ reporter gene
[c]Floxed, loxP-flanked allele
[d]CP-31398, a p53 stabilizing agent
[e]DFMO, difluoromethylornithine, an inhibitor of ornithine decar boxylase

evolution from the earliest stages of tumor initiation to the very advanced stages of tumor progression. With the reductionist approach, mouse models are suitable for defining the biological potential, or the lack thereof, of a specific genetic alteration in tumorigenesis. It is also possible now to generate compound mice harboring multiple genetic alterations, which allow a comprehensive assessment of their combined effects on tumor initiation and progression. Overall, genetically engineered mouse models (GEMMs) have helped (1) establish or refute the in vivo role of many putative genetic alterations that have been identified in humans; (2) differentiate molecular drivers from passengers; (3) discern whether a genotype–phenotype relationship is correlative or causal; (4) validate or disapprove the presumptive collaborative relationships among oncogenes and tumor suppressor genes and between them; (5) define the precancerous lesions, their compensatory tumor barriers and steps that are required to overcome in order for tumors to initiate; and (6) identify divergent progenitor cells for phenotypic variants [8–13]. Examples will be provided in the following sections to illustrate some of these points.

How Do We Model?

Several approaches exit to generate GEMMs. These include transgenic expression of a gene of interest by injection of fertilized eggs with a transgene followed by production of *transgenic mice*; ablation of the function(s) of an entire gene through homologous recombination in mouse embryonic stem cells followed by production of *knockout mice*; alteration of a specific domain or a single amino acid residue with *knock-in mice*; and generation of *compound mice* that involve any combination of the aforementioned approaches. Although most have been well adopted in the field of bladder cancer (Table 13.2), important caveats need to be kept in mind. *First* and foremost, one needs to consider targeting genetic alteration(s) specifically into the urothelium. This is mainly because genetic changes made ubiquitously can cause tumors in vital organs and premature deaths before bladder cancer arises. Case in point, global knockout of p53 tumor suppressor leads to thymic lymphomas and soft tissue sarcomas around 3–7 months of age when urothelium appears completely normal [14]. Global knockout of RB1 is even embryonically lethal [15, 16]. In this regard, the uroplakin II promoter-based urothelium-specific targeting strategies have served the field well [8, 17–19]. The promoter is primarily restricted to the urothelium, as evidenced by the lack of transgene expression in non-urothelial tissues in a wide range of transgenic and knockout lines. It is active in all urothelial layers including the basal layer [18]. It retains its activity even after malignant transformation and in highly advanced bladder tumors [20]. It should be noted that there were erroneous statements in several publications [10, 21–23], all quoting an original source [24], claiming a cloning mistake in the mouse uroplakin II promoter that resulted in the inversion of a 1.5-kB SacI-digested DNA fragment. In actuality, the fragment was only misaligned during sequence assembly and the computing mistake had been corrected in GenBank. There is absolutely no issue with the validity of the promoter per se, hence its continued usefulness. Several non-uroplakin-based

Table 13.2 Existing GEMMs of bladder cancer

Strain designation	Promoter for urothelial targeting	Genetic alteration	Tumor precursor	Tumor morphology	References
Low-grade papillary BC					
UPII-HRAS*/*	Mouse uroplakin II	HRAS* (Q61L)	Simple and nodular hyperplasia	Low-grade, papillary (pTaG1)	[32, 47]
UPII-β catenin* floxed[a] PTEN	Mouse uroplakin II	Activated β catenin* and PTEN deletion	Hyperplasia	Low-grade, papillary (pTaG1)	[85]
UPII-β catenin* UPII-HRAS*	Mouse uroplakin II	Activated β catenin*; HRAS* (Q61L)	Hyperplasia	Low-grade, papillary (pTaG1)	[86]
UPII-β catenin* UPII-KRAS*	Mouse uroplakin II	Activated β catenin; KRAS* (G12D)	Hyperplasia	Low-grade, papillary (pTaG1)	[86]
Msx2rtTA;tetO-Cre β catenin*	Msx2rtTA; tetO-Cre	Activated β catenin*	Hyperplasia	Low-grade, papillary (pTaG1)	[87]
AhCreER floxed PTEN floxed LKB1	Cytochrome P450 promoter	PTEN and LKB1 deletion	Hyperplasia	Low-grade, papillary (pTaG1)	[88]
CAG-ATDC	CMV/β-actin hybrid promoter	ATDC/Trim29 overexpression	Hyperplasia	Low-grade, papillary (pTaG1)	[89]
High-grade papillary BC					
UPII-SV40T/UPII-EGFR	Mouse uroplakin II	SV40T and overexpression of EGFR	Simple and nodular hyperplasia	High-grade, papillary (pTaG2/3)	[69]
UPII-SV40T/UPII-HRAS*	Mouse uroplakin II	SV40T and HRAS* (Q61L)	Nodular hyperplasia	High-grade, papillary (pTaG2/3)	[68]
UPII-SV40T/ UPII-FGFR3*	Mouse uroplakin II	SV40T and FGFR3* (S249C)	Simple and nodular hyperplasia	High-grade, papillary (pTaG2/3)	[45]
Adeno-Cre Floxed RB1 and p130, p107−/−	Adeno-Cre (intravesicle delivery)	Deletion of RB family proteins	ND[b]	High-grade, papillary (pTaG2/3) (some pT1)	[90]

Muscle-invasive BC					
UPII-SV40T (high-copy)	Mouse uroplakin II	SV40T	CIS	MIBC (founder mice only)	[33]
CK19-SV40T	Cytokeratin 19	SV40T	CIS	MIBC	[25]
Floxed Ncstn	Rosa26rtTA;tetO-Cre	Deletion of Nicastrin	CIS	MIBC	[91]
UPII-Cre floxed p53/RB1	Uroplakin II-Cre	p53 and RB1 deletion Plus BBN	CIS	MIBC	[15]
Fabp-Cre floxed PTEN	Fatty acid binding protein-Cre	PTEN deletion	CIS	MIBC (10% in aged mice)	[92]
Floxed p53/PTEN	Adeno-Cre (intravesicle delivery)	p53 and PTEN deletion	CIS	MIBC	[27]
UPII-Cre Floxed p53 UPII-HRAS*	Mouse uroplakin II	p53 deletion plus HRAS* activation	CIS	MIBC (basal subtype)	[52]
CAG-ATDC	CMV/β-actin hybrid promoter	ATDC/Trim29 overexpression	CIS	MIBC	[89]

Notes:
[a]floxed, loxP-flanked allele
[b]*ND* not determined

promoters, such as those of cytokeratins 5 and 19 and fatty acid binding protein (Fabp) [13, 25, 26], have also been used to develop GEMMs. Since these promoters are not urothelium-specific, they are more suited when tumorigenesis is induced, additionally, by a bladder-specific carcinogen [13] or in a “conditional system” where an inducer is delivered intravesically ([27] and see later). The *second* aspect that needs to be taken into account is whether to activate or inactive genetic alterations during embryogenesis or in adult mice. Although the former is more technically straightforward, the latter is preferred because bladder cancer is an adult disease occurring primarily in the aging population. Additionally, altering genes during the embryonic stage when cells are more plastic might trigger unwanted compensatory tumor responses that mask certain phenotypes that would have occurred otherwise [28]. Both tetracycline- and tamoxifen-based, inducible systems have been made available to carry out gene activation and inactivation in adult mice [13, 28, 29]. These systems are expected to be used more extensively going forward. It should be pointed out, however, that the inducible systems are not without any downside. The inducers, particularly tamoxifen, an inhibitor of estrogen receptor, could affect bladder tumor growth and/or progression [28, 30, 31]. The *third* consideration relates to gene dosage. When generating transgenic mice for gene expression, the transgenes are inserted randomly into mouse genome, frequently in more than one copy. The copy number is, however, not an event that can be controlled. Multiple copies are sometime desirable to achieve gene overexpression. This outcome may be accomplished by generating several transgenic lines from which to select the high transgene copy lines [32, 33]. On the other hand, one needs to be aware of whether the degree of over-activation of an oncogene or growth factor receptor is beyond physiological. In this regard, when over-expression is undesirable, one needs to consider creating a point mutation in an endogenous oncogene through the knock-in approach [34] or inserting one-copy of a transgene through homologous recombination into a pre-engineered locus in mouse genome, such as the ROSA26 locus [35]. *Fourth*, one must be mindful of the genetic background, an otherwise major advantage of working with GEMMs but could become a nuisance if experiments are not well planned. It has been fairly well established that the genetic background can have a major influence on the tumorigenic process [36]. For this reason, to facilitate interpretation, it is best to ensure that GEMMs and their non-GEMM controls are generated and maintained in an inbred background. In the situations where mice were previously generated in a mixed background, it is highly advisable to (i) perform continuous backcrossing into an inbred background for at least six generations and/or (ii) generate control groups whose backgrounds are as close to experimental groups as possible. *Finally*, there is emerging evidence suggesting that different urothelial cell layers might serve as different progenitors for bladder cancer variants [13, 37]. In mice, basal, intermediate, and superficial (umbrella) layers comprise the urothelium. To resolve the issue of urothelial layers vis-à-vis origins of different variants, it will be necessary to target genetic events into different compartments using layer-specific promoters. Since the known layer-specific promoters, such as Krt5/14 for basal layer and Krt20 for superficial layer, are not urothelium-specific, additional efforts are required to isolate new promoters or to manipulate the promoter activities locally.

What Have We Learned?

Low-Grade Papillary Tumors (pTaG1)

A strong consensus exists, based on genetic analyses of human specimens in multiple independent cohorts, that activating mutations in the RTK/RAS/PI3K pathway are highly prevalent in these tumors [8, 38, 39]. The list of genes affected includes FGFR3, FGFR1, ERBB2, HRAS, KRAS, PI3K, BRAF, and RAF [40–42]. Tumor suppressor genes whose inactivating mutations can lead to indirect activation of the pathway include PTEN, TSC1/TSC2, and NF1 [43]. Interestingly, mutations of the different genes within this pathway are mostly non-overlapping [43, 44], suggesting the lack of selective advantage of having simultaneous mutations in two genes in the same pathway. These results have largely been confirmed and extended by recent whole-genome and whole-exome sequencing [40–43]. Overall, 80–90% of the low-grade papillary tumors are estimated to harbor at least one gene mutation in this pathway.

Does the exceedingly high frequency of mutations in RTK/RAS/PI3K pathway foretell their strong urothelial tumorigenicity or their being bona fide drivers of this bladder tumor variant? The answer is surprisingly no. On the contrary, these mutations by themselves have very limited tumorigenic potential as evidenced by multiple GEMM studies [38]. For instance, transgenic expression of an S249C mutant of FGFR3, the most prevalent of all the FGFR3 mutations in human BC, did not induce any urothelial proliferation, let alone tumors [45]. Similarly, mice expressing a K644E mutant that constitutively activates the kinase domain of FGFR3 were also tumor-free after extended follow-up [46]. Ablation of both PTEN alleles produced urothelial hyperplasia, with only a small percentage (10%) of the mice developing low-grade papillary BC in aged animals [26]. An HRAS mutant engineered through the knock-in approach and expressed from its endogenous promoter, thus avoiding overexpression, failed to produce any urothelial abnormality over a year [34]. A major exception to these examples is the interesting observation from the transgenic mice bearing a constitutively active HRAS under the control of the mouse uroplakin II promoter [32]. The heterozygous offspring exhibited simple urothelial hyperplasia up to 10 months of age, while all of the homozygous offspring developed low-grade, papillary BC by 3 months of age [47]. Therefore, not only is the quality (mutational activation per se) but also the quantity (level of mutant expression) critical for an oncogene such as HRAS to serve as a tumor driver. This is not to say that mutated oncogenes cannot be tumorigenic in the absence of high dosage. Tumorigenesis may still ensure if there is a collaborative partner, such as the deficiency of a tumor suppressor gene. Deletion of Ink4a, a frequent event in low-grade papillary BC that was shown to cooperate with RAS activation in non-urothelial tissues, has been ruled out as a collaborative partner [47]. This is because removal of Ink4a from transgenic mice expressing a low-level of HRAS in urothelial cells did not produce bladder tumors. So, in essence, RAS mutation and, for that matter, mutations of the other components in the RTK/RAS/PI3K pathway are in still search of a partner or a co-driver [38].

High-Grade Muscle-Invasive Bladder Cancer (pT1–4)

A major surprise from the GEMM studies in terms of the putative genetic drivers for muscle-invasive bladder cancer (MIBC) was the complete lack of tumorigenicity from the loss of both p53 and RB1 in two independent studies using different approaches [15, 27]. Given the fact that concurrent p53 mutations and aberrant expression of RB1 were among the most prevalent genetic alterations in human MIBC [48–50], it was entirely unexpected that ablation of both p53 and RB1 genes from the embryonic stage or adult mice failed to produce any urothelial lesion, not to mention MIBC. The double knockout mice were nevertheless more susceptible to bladder-specific carcinogen *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in developing MIBC than the single knockout mice lacking either p53 or RB1 [15]. The absence of spontaneous tumors and the susceptibility to bladder-specific carcinogen of p53/RB1 double knockout mice suggests that the loss of these two major suppressors is necessary but insufficient to induce MIBC.

Two recent studies pointed to a different scenario for MIBC genesis. Instead of RB1 deficiency as a co-driver, p53 deficiency may actually synergize with RTK/RAS/PI3K pathway activation to drive invasive urothelial tumorigenesis. Puzio-Kuter and colleagues first obtained double transgenic mice in which functionally critical regions of both p53 and PTEN were flanked by loxP sequences (double floxed mice) [27]. To delete the two genes in adult mice, the investigators introduced an adenovirus-driven Cre recombinase directly into the adult mouse bladders via surgery. Mice deficient for both p53 and PTEN, but not for each gene alone, developed early-onset carcinoma in situ (CIS) and MIBC. Consistent with PTEN acting as a potent inhibitor of PI3K, its loss resulted in marked activation of PI3K downstream effectors in the double knockout mice [27]. The investigators took a step further to show that inhibition of mTOR, a downstream effector of PI3K pathway, by rapamycin greatly reduced the occurrence of MIBC in p53/PTEN double deficient mice [51]. In a separate study, He and colleagues created a compound mouse model in which the urothelial cells express an activated HRAS and simultaneously lack both alleles of p53. These mice, like the p53/PTEN double knockouts, developed CIS and MIBC with relatively early-onset and high penetrance [52]. Both aforementioned models bear strong resemblance to the MIBC in humans in phenotypes and mode of progression (e.g., from CIS to invasion). These results are highly significant in light of the recent findings from the TCGA study indicating that the genetic alterations affecting the RTK/RAS/PI3K and the p14-MDM2-p53-p21 occur in 72% and 76% of human MIBC, respectively [40]. Therefore, even by genetic drifting, over half of the human MIBC would harbor one event from each pathway. This is an excellent example where mouse modeling and human studies are pointing in the same direction with respect to the genetic drivers of bladder cancer. Further studies are clearly needed to sort out the more detailed collaborative relationships between the different components of these two pathways.

Phenotypic Subtypes of MIBC

Recent molecular phenotyping revealed that human MIBC is not a single entity but can be classified into different subtypes [20, 53–59]. At the minimum, there are three major subtypes [53, 60]. The luminal subtype is characterized by phenotypes expected of the luminal or superficial cells of the normal urothelium, such as abundant cytokeratin 20, uroplakins and E-cadherin. Commonly mutated genes include fibroblast growth factor 3 (FGFR3) and tuberous sclerosis 1 (TSC1). The basal subtype is characterized by markers normally expressed by the urothelial basal cells, such as cytokeratins 5 and 14, CD44 and markers of epithelial-mesenchymal transition (TWIST1/2, SNAI2 and ZEB2). The basal subtype also frequently coexists with squamous elements of epithelial transdifferentiation, and is clinically more aggressive and chemoresistant than the luminal subtype [53, 60]. Both the luminal and basal subtypes carry frequent p53 pathway mutations. The third subtype was designated as "p53-like MIBC" that resembles the luminal subtype in molecular phenotyping but differs from it in its bearing a wild-type p53 [53, 60]. Somewhat counterintuitively, this subtype is more resistant to chemotherapy than mutant-p53-bearing MIBCs. This is probably due to the fact that the wild-type p53 is a key DNA-repair regulator whose deficiency renders tumor cells less capable of repairing DNA damages caused by certain chemotherapeutics than wild-type p53-expressing tumor cells. It should be noted that the net effects of p53 deficiency on increasing the sensitivity to chemotherapeutics while also increasing the likelihood of tumor progression needs to be sorted out. This issue applies to mutations in other DNA repair enzymes such as ERCC2 recently identified through whole-genome/exome sequencing [61, 62]. GEMMs should play a role in this regard.

Thus far, the basal subtype MIBC has been recapitulated in GEMMs. As mentioned above, a combination of activated HRAS and homozygous deletion of p53 led to MIBC [52]. By immunohistochemistry, the invasive lesions express high levels of basal cell-specific cytokeratins 5 and 14 and stem cell marker CD44 and reduced level of cytokeratin 20. They also exhibit histological features typical of focal squamous differentiation and express abundant cytokeratin 1 and Trim29, markers for squamous metaplasia [52]. Therefore, the experimental evidence from this GEMM model raises the interesting possibility that the defects in RTK/RAS/PI3K and p14-MDM2-p53-p21 cooperate to provoke basal-subtype MIBC. The histological features of MIBC in the p53/PTEN double knockout model were not clearly delineated and differentiation markers not assessed [27]. It would be of interest to see if they also belong to the basal subtype, which would lend further support to the dual-pathway concept proposed here.

Major questions regarding MIBC subtypes remain that GEMMs should be helpful in ascertaining. For instance, do different MIBC subtypes have different genetic drivers? Are the subtypes interchangeable through tumor cell dedifferentiation

during progression or therapeutically induced differentiation? What are interrelationships between MIBC subtypes and non-invasive BC? It is well known that 10–30% of non-muscle-invasive BC progress to MIBC [63, 64]. When they do progress, which MIBC subtype do they become? Answers to these questions should be forthcoming in the next few years with significantly expanded efforts on models that incorporate an increasing number of recently identified genetic alterations from the whole-genome analyses.

Progenitor Cells and BC Variants

In the absence of direct experimental evidence, all BC variants were presumed in the past to originate from a single stem/progenitor cell. Recent lineage tracing studies using GEMMs are starting to challenge this old paradigm. The approach entails the indelible marking of potential progenitor cells with reporter genes controlled by cell-type-specific gene promoters such as cytokeratin 5 for basal cells and uroplakin IIIa for intermediate and superficial cells [13]. After these cells are permanently marked by short-term, pulse treatment of a chemical such as tamoxifen, the host mice are treated with a bladder-specific carcinogen, BBN. The contribution of differently marked cells to BBN-induced tumor lesions is then determined based on reporter gene expression. These techniques that were used extensively to identify lineage-specific progenitors in other organ systems had not been used for BC until recently. Van Batavia and colleagues found, using the said BBN carcinogenesis method, that the uroplakin promoter-marked cells contributed primarily to non-invasive papillary bladder tumors [13]. In contrast, Krt5-marked cells contributed mainly to CIS and MIBC, the latter exhibiting prominent squamous features [13]. Another study from Beachy's group indelibly marked the basal cells with a sonic hedgehog promoter and arrived at a similar conclusion that urothelial basal cells are the progenitor cells of CIS and MIBC [37]. Results from these studies therefore raise the interesting prospect that different progenitor cells may form different BC phenotypic variants.

Notwithstanding the exciting new information, further studies are warranted to study whether BC variants are caused by different genetic drivers or different progenitor cells, or both. The existing lineage-tracing models all make use of BBN to induce BC [13, 37, 65]. One therefore cannot completely rule out the possibility that at least some of what have been observed might apply only to this particular carcinogen. Introducing specific genetic alterations via genetic engineering into lineage-tracing models should alleviate some of the concerns. Because of a close relationship between normal and cancer stem cells, a better understanding of the normal urothelial stem cells should also help better define the cell(s) of origin of BC variants. Finally, addressing the issue of BC heterogeneity and clonality will be greatly facilitated by single-cell sequencing of human BC specimens. Information obtained can be fed back into GEMMs for validation.

High-Grade Papillary Tumor (pTaG2/3)

This remains as an enigmatic variant in humans with respect to its biological behavior and genetic underpinning [66, 67]. In GEMMs, urothelial expression of an SV40 large T antigen (functionally disabling p53 and RB1), plus activation of one of components in the RTK/RAS/PI3K pathway, consistently yields this phenotypic variant. The low-copied uroplakin II-SV40T transgenic mice by themselves develop CIS lesions at young ages (2–10 months of age) that slowly progress to high-grade non-invasive papillary BC (pTaG2/3). This process can be accelerated significantly by co-expressing in the urothelial cells an activated HRAS (tumorigenesis by 2–3 weeks) [68], or activated FGFR3 (by 5–7 months) [45], or overexpression of EGFR (by 5–8 months) [69]. Thus, the high-grade non-invasive papillary BC (pTaG2/3) may just be a result of high-grade, flat CIS lesions gaining additional growth potential from activation of RTK/RAS/PI3K components. This is consistent with the fact that pTaG2/3 BC often co-exist with CIS lesions in humans [70, 71]. Based on the genetic alterations (both p53/RB1 and RTK/RAS/PI3K pathways) causing this variant in GEMMs, it reinforces the idea that this variant needs to be managed more aggressively than its low-grade counterpart.

What Might Be a Balanced View of Mouse Modeling?

While mice and humans share striking similarities, the former are not a miniature version of the latter and significant differences exist. There are ample examples where the principles firmly established in mice did not pan out in patients [72]. GEMMs are no exception. It is incumbent for the developers as well as the users to be cognizant of the pros and cons of the specific model(s) at hand. Simply put, no model is perfect but many are useful. The key is whether one asks the right questions with the right models. This view should not be limited to GEMMs, but should encompass all the model systems as a whole.

To make GEMMs as useful and human-relevant as they can be, the modeler should perhaps start with genetic alterations that are highly prevalent in human BC, thus likely serving as a driver or co-driver of a particular phenotypic variant. The modeler should also be mindful of the fact that a single genetic hit from each oncogenic or tumor suppressor pathway is unlikely to be tumorigenic and two or multiple hits from divergent signaling pathways may be necessary and sufficient for tumor initiation. Another important aspect is about data interpretation; one should always compare and contrast phenotypic alterations observed in mice, whether they be precancerous lesions, begin tumors, advanced tumors and tumor subtypes, with the human counterparts. Consultation with or direct involvement of genitourinary pathologists, urologists, and oncologists is critical. Last but the least is the necessity of validation of any and all results from GEMMs with human materials. The importance of data validation cannot be overstated. Information flowing

from humans to mice and then from mice to humans is the only way to ensure the "fidelity of human-to-laboratory models."

Finally, modeling of BC with GEMMs has moved considerably beyond simply generating a toolbox for testing new diagnostics, therapeutics, and preventive strategies (Table 13.1). When viewed as a toolbox, the more closely the models resemble their human counterparts, the more useful they become. However, when the modeling is used to define the genetic and epigenetic drivers and dissect the sequential steps of tumorigenesis, the burden of mouse-human resemblance becomes less stringent. More often than not, barriers become opportunities. For instance, the lack of an expected phenotype from a specific genetic alteration means the need to test additional alterations or a different set of alterations. The confinement of tumorigenesis in the precursor stages actually offers opportunity for identifying tumor barriers and how overcoming the barriers may enable tumor initiation. By the same token, the generation of low-grade tumors may allow the identification of drivers for tumor progression. In short, challenges can be turned into opportunities when it comes to GEMM modeling.

What Does the Future Hold?

The next few years will likely see a considerable expansion of GEMMs, both in breadth and depth, for BC. Whole-genome and whole-transcriptome profiling is being performed on an increasing number of BC patients encompassing different phenotypic variants. Single-cell exome and RNA sequencing should also come to the forefront, yielding unprecedented amounts of information on inter- and intra-tumor heterogeneity, clonal expansion, stem/progenitor cell genetic landscape and lineage evolution [2, 73]. A consensus will likely emerge on recurrent genotypes and phenotypes on each BC variant. The recurrent genetic alterations need to be validated in GEMMs for their driver potential, their roles in tumor initiation and progression, and their collaborative relationships with other genetic or epigenetic alterations during the multistage tumorigenesis. The ongoing work at the International Knockout Mouse Consortium (http://www.mousephenotype.org/about-ikmc), whose goal is to knock out every protein-coding mouse gene, should greatly facilitate this process. Nevertheless, the responsibility of generating transgenic mice overexpressing oncogenes and growth factor receptors and knock-in mice with domain- or site-specific mutations still rests with the individual modelers. This is also true with the generation of compound mice bearing multiple engineered alleles. It is possible, but appearing unlikely, that the number of the alleles required for BC initiation and progression will reach a level beyond what can be handled technically.

The next-generation GEMMs of BC will also see a transition from more traditionally constitutive models (e.g., embryonic expression or ablation) to inducible ones (e.g., gene changes in adult mice). There will be an increased popularity of the

use of new genome editing techniques such as CRISPR/Cas9 [74]. Targeting of genetic alterations into urothelium will involve more precision, from the whole urothelium to different compartments/layers/lineages. The GEMMs of BC are here to stay and the future for using them to study and treat BC is bright.

Conflict of Interest
The author declares no conflict of interest.

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