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TOPIC PAPER

SIU–ICUD consultation on bladder cancer: basic science

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Abstract

Purpose To provide a condensed summary of the Basic Science chapter that was included in the Third International Consultation on Bladder Cancer.

Methods World bladder cancer basic science experts used the published literature to create summaries of recent progress in their areas of expertise.

Results The completion of several large-scale genomics projects coupled with a strong collaborative culture within the research community and the exciting clinical activity of immune checkpoint blockade have combined to transform the bladder cancer research landscape. Bladder cancer molecular subtypes and the presence of specific DNA alterations provide important information about disease heterogeneity that has direct implications for clinical management, and some can be targeted by compounds that are already clinically available. Tests are being developed that can measure many of these alterations non-invasively in peripheral blood or urine, raising confidence that they could be used as biomarkers for surveillance and monitoring the effects of local and systemic therapies.

Conclusions Although the bulk of the mechanistic work lies ahead, the genomics results have created a hypothesis-generating description of bladder cancer heterogeneity that has set the stage for deeper mechanistic studies, and they have already provided us with extremely attractive candidate biomarkers to guide clinical practice. Here, we will summarize the recent progress in basic bladder cancer research and highlight near-term opportunities for the future.

Keywords Bladder cancer · Molecular subtypes · Immunotherapy · DNA damage and repair mutations · Targeted therapy · Preclinical models

Introduction

Urothelial cancers of the bladder (bladder cancer) can be subdivided into two major disease states with different implications for clinical management [1, 2]. Non-muscle-invasive bladder cancers (NMIBCs) correspond to the bulk of cancer incidence. They generally do not pose a significant threat to the life of the patient but do invariably recur, necessitating expensive lifelong cystoscopy and local resection that generate significant patient discomfort and make NMIBC the most expensive of all cancers to clinically manage. Importantly, a fraction of high-grade NIMBCs do progress to become invasive, but no tools are available to prospectively identify these tumors, and surgeons must rely on their clinical judgment and experience to decide when to offer patients definitive therapy. On the other hand, muscle-invasive bladder cancers (MIBCs) are clinically aggressive, and up to 50% of patients die of their disease. Again, there are no tools that can be used to distinguish patients with lethal cancers from those that can be cured.

Pathologists have long recognized that bladder cancers also exhibit distinct growth patterns [3]. Most NIMIBCs exhibit papillary growth patterns, whereas most MIBCs appear to progress from flat lesions, including carcinoma in situ (CIS) [3]. Early genomic studies demonstrated that papillary tumors are enriched with activating mutations in fibroblast growth factor receptor-3 (FGFR3), and indeed up to 80% of papillary NMIBCs harbor these mutations [2]. Early genomic studies also demonstrated that tumors in the non-papillary pathway are enriched with inactivating

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mutations in *TP53* and *RB1* [2], and not surprisingly, RNA expression profiling studies demonstrated that the papillary and non-papillary tumors are very different at the gene expression level [4, 5]. With this work as a foundation, the genomic advances that have been made over the past 5 years have provided a much more granular depiction of bladder cancer heterogeneity and have provided important insights into the pathogenic processes that underlie it.

Molecular subtypes of bladder cancer

As introduced above, early efforts to use mRNA expression profiling to define bladder cancer heterogeneity revealed that papillary, non-muscle-invasive cancers could always be distinguished from non-papillary, muscle-invasive cancers [4–6]. Subsequent work led by the group at Lund demonstrated that combined cohorts of NMIBCs and MIBCs could be subdivided into at least five separate clusters, each endowed with gene expression features suggestive of specific biological processes [7] (Fig. 1). "Urobasal A" (now termed "urothelial", uroA) cancers were enriched with activating *FGFR3* mutations and FGFR3- and early cell cycle-related gene expression signatures and were associated with the best clinical prognoses. "Urobasal B" (uroB) tumors shared these FGFR3-related features, but displayed lower expression of early and higher expression of late cell cycle genes. Like uroA, the "genomically unstable" (GU) tumors expressed urothelial terminal differentiation markers, but unlike uroA, they displayed higher percentages of *TP53* mutations, late cell cycle gene expression signatures, and, perhaps as a consequence, shorter disease-specific survival. "Infiltrated" tumors were characterized by heavy infiltration with myofibroblasts and other stromal cells (including T cells) and extracellular matrix (ECM) gene expression signatures, and "squamous cell carcinoma-like" (SCCL)

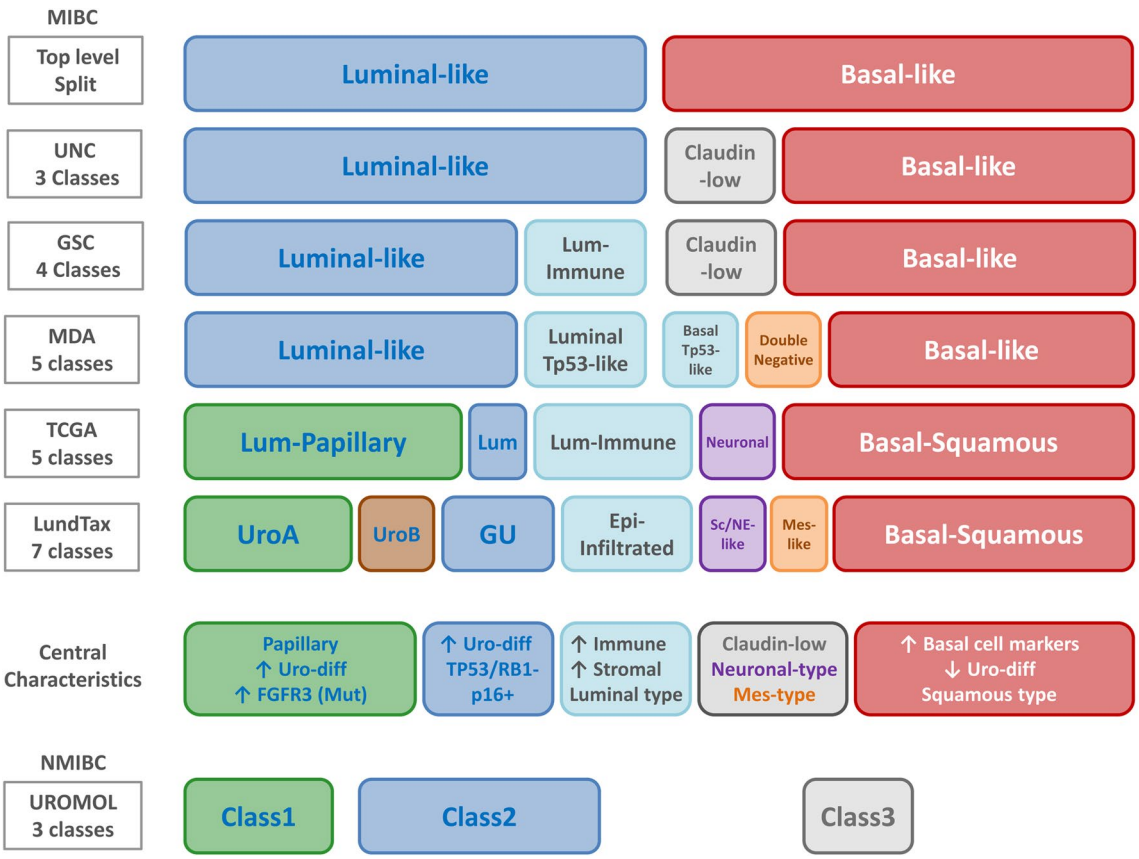

Fig. 1 Molecular subtypes of bladder cancer. Whole transcriptome RNA expression profiling has revealed the existence of distinct bladder cancer molecular subtypes. At the highest level, MIBCs can be divided into luminal- and basal-like subtypes based on differential expression of biomarkers associated with terminal differentiation. These subtypes have been further subdivided by groups at the University of North Carolina (UNC), the University of British Columbia in collaboration with GenomeDx (GSC), MD Anderson Cancer Center (MDA), The Cancer Genome Atlas (TCGA), or Lund University (LundTax) based on the presence or absence of central characteristics that reflect important differences in tumor biology, some imparted by stromal cells. The molecular subtypes of NMIBCs were also identified by the UROMOL consortium (classes 1–3), and they possess some of the central characteristics that were associated with the subtypes of MIBCs. Reprinted with permission from the International Consultation on Urological Diseases (ICUD)

tumors contained squamous biomarkers, were enriched with squamous histological features and, together with uroB, the shortest disease-specific survival.

Since this pioneering work was published there has been strong interest in further defining the molecular subtypes of bladder cancer. Led by TCGA, several North American groups performed independent analyses of whole transcriptome datasets obtained from MIBCs and concluded that cancers could be divided into basal and luminal subtypes that shared features with corresponding subtypes of breast cancers [8–10] (Fig. 1). More recently, attempts were made to reconcile the original Lund subtypes with those defined by the other groups [11, 12], revealing strong consensus—uroA and GU tumors largely correspond to the "luminal" subtypes defined by the other groups, whereas the uroB and SCCL tumors are usually assigned to the basal subtypes. A fraction of the basal tumors display gene expression patterns consistent with epithelial-to-mesenchymal transition (EMT) [8, 9, 12], and these tumors resemble the so-called "claudin-low" subtype of breast cancers [13]. Finally, parallel efforts by the Lund group [11] and TCGA [14] revealed the existence of another subset of basal cancers characterized by the expression of neuroendocrine biomarkers and aggressive clinical behaviors. The idea that the bladder cancer variants may display strong basal/luminal subtype bias is supported not only by the observation that squamous and neuroendocrine/small cell features are enriched in basal cancers [7, 8, 10, 11, 14], but also that almost all micropapillary [15] and plasmacytoid [16] cancers cluster with the "luminal" cancers. Whole transcriptome analyses of non-muscle-invasive bladder cancers (in the UROMOL study) revealed the existence of three clusters (classes) that shared some of the central characteristics that were observed in the MIBCs [17] (Fig. 1).

Another work indicates that the molecular subtypes are associated with differential benefit from chemo- and immunotherapy. One of the original studies demonstrated that the "p53-like" subtype (corresponding largely to the Lund infiltrated tumors) was associated with chemoresistance [10], and subsequent studies demonstrated that patients with basal tumors derived the most benefit from cisplatin-based chemotherapy [18, 19]. Conversely, patients with "infiltrated" and in particular Lund GU tumors appear to obtain more benefit from immune checkpoint blockade with anti-PD-L1 antibodies [20, 21]. The relationships between molecular subtype membership and benefit from chemotherapy are currently being prospectively evaluated in tumors collected from an important Phase II clinical trial (S1314, led by the US Southwest Oncology Group) [22]. In parallel, several groups are using what are now publicly available datasets to further explore the relationships between subtype membership and benefit from immunotherapy. Physicians also have access to a molecular diagnostic test that can be used to assign tumors to the molecular subtypes (Decipher, from GenomeDx) [19], so molecular subtyping is now being integrated into some clinical trials. It should not be long before the published preliminary findings have been subjected to validation.

Studies led by TCGA have also demonstrated that non-coding RNAs, including micro RNAs and long non-coding RNAs (lncRNAs), can also be used to visualize bladder cancer molecular subtypes [8, 14, 23]. Indeed, these subtypes largely correspond to those defined at the mRNA level, but they also represent important subjects for further mechanistic interrogation. In addition, the stability of micro RNAs in body fluids could potentially be exploited for molecular subtyping in blood and urine. Further efforts to define the molecular subtypes of NIMBC are also underway, but the Lund observation that they tend to be enriched with uroA and GU (luminal) tumors has already been confirmed.

There are interesting biological relationships between the gene expression signatures that are present in basal and luminal tumors and the transcriptional control of normal urothelial development. The latter contains a basal layer that is characterized by expression of many of the same biomarkers that are expressed by basal tumors, and the more well-differentiated layers of the urothelium are characterized by expression of bladder cancer luminal biomarkers [24]. Preclinical studies in normal urothelial organ cultures defined a central role for peroxisome proliferator activator receptor-gamma (PPARG) in the control of normal urothelial differentiation [25–27], and PPARG expression and downstream target gene expression are also characteristic features of luminal tumors [10, 28]. Conversely, the basal layer is characterized by the expression of the epidermal growth factor receptor (EGFR) [24], and studies in primary urothelial cultures demonstrated that EGFR signaling inhibits PPARG-mediated differentiation [25]. Ongoing in vitro and in vivo experiments in normal human urothelial cells and mouse models have been exploring the roles of these and other pathways in the control of differentiation, the response to bacterial infection, wound healing, and carcinogenesis. There is little doubt that a better understanding of normal urothelial physiology and plasticity will shed light on similar transcriptional mechanisms that control similar processes in bladder cancers.

Cancer stem cells

Accumulating evidence highlights the importance of intratumoral cellular heterogeneity in human bladder urothelial carcinomas, which harbor functionally distinct cancer stem cells (CSCs; or tumor-initiating cells). These CSCs are enriched with tumor-initiating potential when engrafted in vivo. A panel of CSC markers has been reported, which includes, but is not limited to, CD44, CD44v6, CD49f, CD90, CD133, 67LR, ALDH1, CD14, and KRT14

[29–36]. Basal subtype tumors express CSC markers, e.g., CD44, CD49f and KRT14, while luminal subtype tumors are associated with relatively lower expression of these markers. Basal tumors with high expression of CSC markers are associated with poor overall survival in a treatment-naïve setting, implicating their prognostic value.

State-of-the-art single cell exome sequencing of paired CD44(pos) cancer/normal stem cells versus CD44(neg) differentiated cells suggests that CSCs could clonally arise from either normal stem cells or differentiated cells [37]. Further functional analysis revealed that mutations in MLL2 co-operate with ARID1a and GRPC5a mutants, thereby significantly augmenting sphere-forming and tumor-initiating properties [37]. Another study revealed that a C228T mutation of the TERT promoter frequently occurred in CSCs, but not in differentiated cells [38]. Such findings echo TCGA and other high-throughput analysis studies, as MLL2 (27%), ARID1a (25%) and TERT are among the most frequently mutated genes in human bladder urothelial carcinomas [8, 14, 36, 39, 40], independently supporting the functional significance of CSCs in the pathogenesis of bladder cancer development. The transcription factor STAT3 was involved in the regulation of CSC expansion [41], which corresponded with rapid development of CIS and invasive progression. A recent study connected a mechanistic link of KMT1A–GATA3–STAT3 in regulating self-renewal of bladder CSCs [42]. The histone methyltransferase KMT1A augmented H3K9me3 modification on the gene promoter of GATA3 in bladder CSCs, suppressing transcriptional activity of GATA3 [42]. This in turn activates STAT3 activity, since GATA3 is a transcriptional suppressor of STAT3 [42]. In addition to cell intrinsic regulation of CSCs, studies using elegant mouse models shed light on the cross talk between stem cells and stroma during bladder cancer progression. Urothelial basal stem cell expression of sonic hedgehog (SHH) was found to induce stromal-derived factors BMP4 and BMP5, which were paracrine inducers of urothelial differentiation that restrained invasive progression [43]. When SHH was lost during tumor progression, stromal-derived factors restrained invasion were also lost, therefore facilitating tumor invasion [43].

Cancer stem cells are intrinsically less responsive to conventional chemotherapy due to several protective properties that CSCs share with normal stem cells. These include higher expression of drug-efflux pumps, better DNA repair capacity, and enhanced protection against reactive oxygen species. CSCs can also respond to chemotherapy-induced cell death and associated injury signals, by activating a wound response to "repopulate" residual tumors [44]. Recurrent repopulation of CSCs in consecutive chemotherapy treatment cycles led to increase in CSC content, and thus nonresponsive tumors [36].

Mutational processes in bladder cancer

Parallel efforts by TCGA and private groups have also transformed our understanding of bladder cancer heterogeneity at the DNA level [2]. The work confirmed that inactivating mutations in *TP53* are common in MIBCs and revealed that inactivating mutations in chromatin-modifying enzymes such as *KMT2D*, *KDM6A*, and *ARID1A* are almost as prevalent (Fig. 2). Even though smoking is a well-established risk factor for bladder cancer, whole exome sequencing failed to reveal direct smoking-related mutational signatures in the TCGA bladder cancer cohort of 408 cancers [8, 14]. Rather, MIBCs are dominated by mutational signatures generated by the APOBEC family of cytidine deaminases, which play important physiological roles in responses to viral infections [45]. In addition, tumors from smokers appear to be enriched with mutational signatures related to inactivation of *ERCC2* [46], a DNA repair protein that is involved in nucleotide excision repair (NER). Importantly, mutational inactivation of *ERCC2* occurs in approximately 9–15% of MIBCs (Fig. 2), and these mutations are associated with response to neoadjuvant cisplatin-based combination chemotherapy [47, 48]. In addition, inactivation of a variety of other DNA damage and repair (DDR) genes, most notably *RB1*, *ATM*, and *FANCC* [49] (Fig. 2 and not shown), has also been linked to chemotherapy sensitivity. Again, the S1314 trial will allow for prospective validation of these potentially clinically impactful findings, and clinical trials have already been designed and opened to determine whether patients whose tumors contain DDR mutations might be cured by neoadjuvant chemotherapy alone. Other studies have used whole exome and DNA panel sequencing to characterize the clonal relationships between tumors collected before and after neoadjuvant chemotherapy, primary tumors and recurrences, and multifocal cancers [50–52]. In general, all of the studies reinforce the conclusion that bladder cancers have clonal origins, but they also reveal the presence of marked heterogeneity caused by clonal evolution that has important implications for acquired resistance and the use of biopsies to guide the selection of targeted therapies.

Expanded efforts to characterize the DNA-based genomic properties of NMIBCs are also underway (Fig. 2). A recent high impact study focused on non-invasive (Ta) tumors revealed the presence of two DNA-based subtypes linked to specific chromosomal abnormalities and levels of genomic instability [53], and DNA mutations targeting *FGFR3*, *KDM6A*, *STAG2*, and *PIK3CA* are much more prevalent in NIBCs than in MIBCs (Fig. 2). Another work has shown that high-grade T1 NMIBCs exhibit DDR mutation frequencies that rival those observed in MIBCs [54], suggesting that cisplatin-based combination

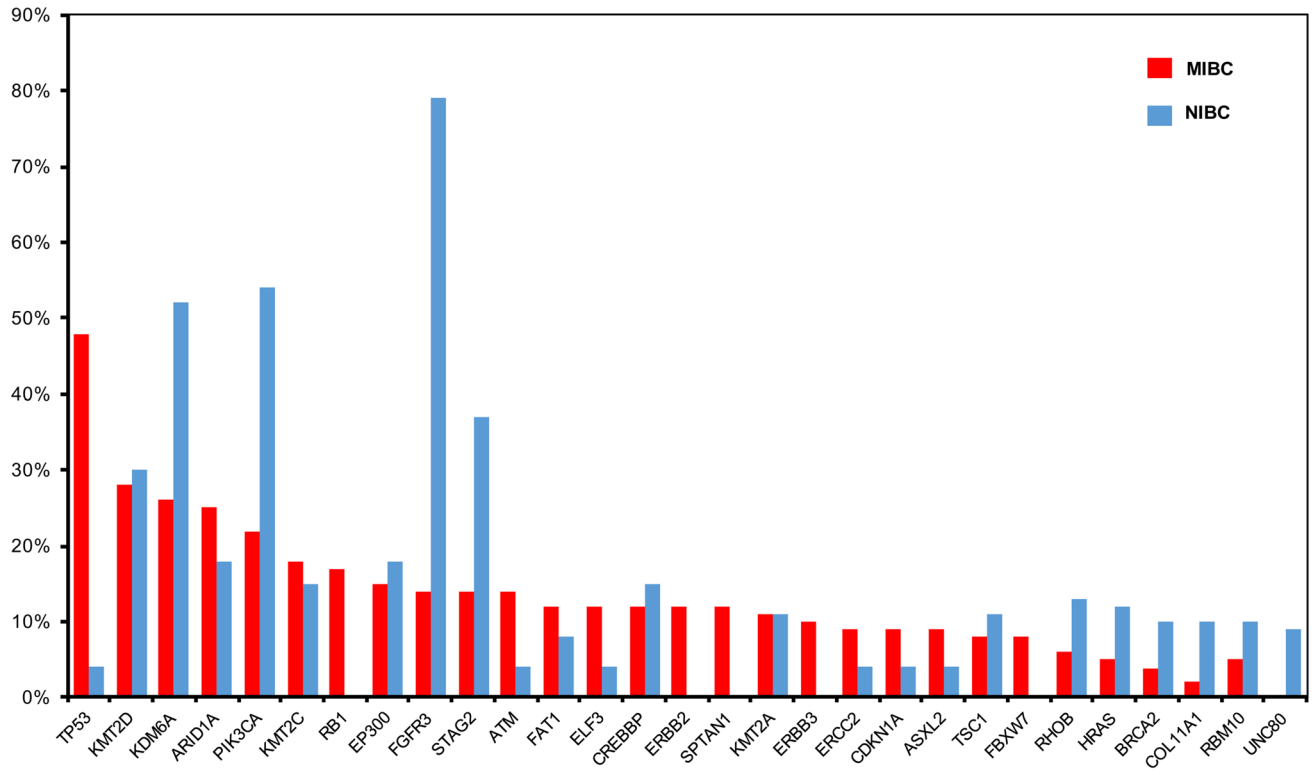

Fig. 2 Prevalence of DNA mutations in MIBCs and NIBCs. The data supporting this figure were derived from Robertson et al. and Hurst et al. [14, 53]. Reprinted with permission from the International Consultation on Urological Diseases (ICUD)

chemotherapy may be active in these tumors. They also appear to have high TMBs and may therefore be recognized by the immune system. Indeed, preliminary results from a relatively small tumor cohort revealed a relationship between higher TMBs and benefit from intravesical BCG [55]. Ongoing studies are testing this hypothesis aggressively and characterizing the mutational and immune landscapes in tumors that acquire resistance. In addition, an FDA registration trial is open (SWOG 1605) to explore a possible role for immune checkpoint blockade in BCG-unresponsive disease. Tumors from this trial will be subjected to whole exome sequencing to determine whether the relationship between TMB and/or DDR mutations or APOBEC signatures are associated with benefit in this earlier disease setting.

Recent studies have demonstrated that the mutations that are present in primary tumors and metastases can be detected in blood and urine, presumably as a result of tumor cell death and lysis [56]. Tumor DNA and RNA can also be found in excreted microvesicles termed "exosomes", and it is possible that exosomes are even highly enriched with tumor as opposed to normal material [57]. This recognition has prompted the development of sensitive techniques to monitor mutations and other genomic abnormalities in "liquid biopsies" [56]. Although optimization of such techniques is still underway, retrospective studies have proved that such methods are highly feasible and potentially valuable as tools for surveillance and selection of therapy [58–61] (Fig. 3). Interestingly, experience to date suggests that, even though tumor DNA is probably more abundant in urine (particularly in patients with NIMBC), tumor DNA tests appear to be more accurate when using serum or plasma as the starting material. Having accurate methods to monitor tumor DNA in both fluids could have complementary roles in patient management. For example, a plasma assay could be useful in selecting systemic therapy and for monitoring responses in advanced disease, whereas a urine assay might be more sensitive for detecting recurrences in patients with NIMBC.

Chromatin modifiers

Early observations from Gui et al. [39] and the TCGA revealed the high frequency of mutations in genes associated with histone modification and chromatin structure in human bladder cancer [8]. Unlike transcriptional activators that directly regulate gene expression, chromatin modifiers may influence promoter or enhancer activity on close (cis regulation) or distant (trans) genes (Fig. 4). Therefore, the

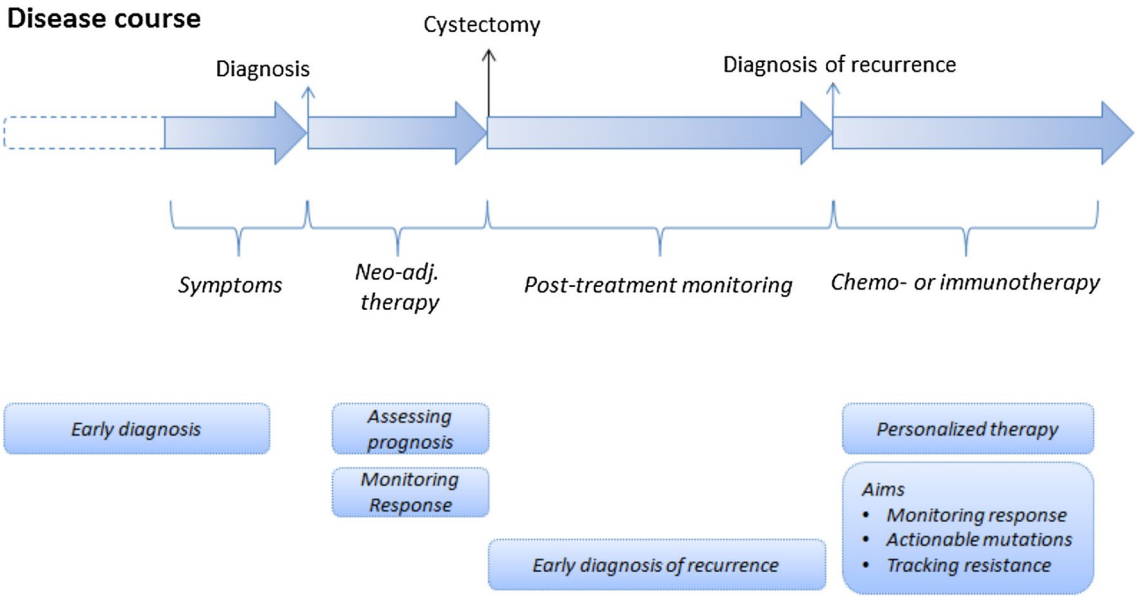

Fig. 3 Opportunities for integrating "liquid biopsies" into the clinical workflow. The detection of DNA mutations in urine could augment assessments of hematuria to aid in early diagnosis. Circulating tumor DNA could be used to detect the presence or absence of DDR mutations and predict benefit from neoadjuvant chemotherapy, and measurements post-therapy could be used to monitor the presence of subclinical local and metastatic disease. Tests for cancer mutations in urine and blood could be used as tools for early detection of recurrence and design of treatment regimens based on tumor molecular properties (for example, the presence of high TMB and/or *FGFR3*, *ERBB2*, or *ERBB3* mutations). Reprinted with permission from the International Consultation on Urological Diseases (ICUD)

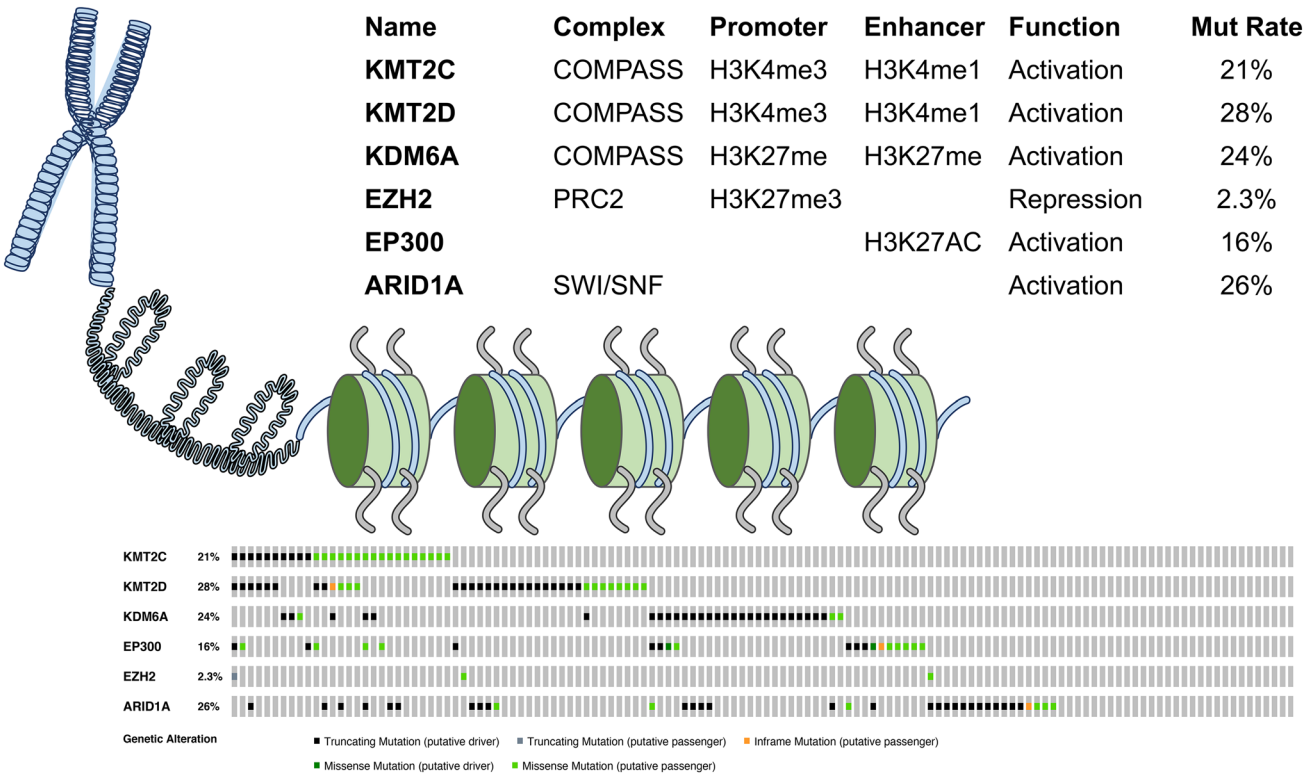

Fig. 4 Frequencies of chromatin-modifying gene mutations in bladder cancer. The table (top) lists the major affected chromatin-modifying genes and their enzymatic activities. The figure (bottom) displays the types of mutations found in the final TCGA dataset and their associations with one another. Reprinted with permission from the International Consultation on Urological Diseases (ICUD)

function of individual chromatin modifiers may be pleiotropic depending on the cellular context.

Advances in next-generation sequencing and chromatin immunoprecipitation have facilitated the identification of genomic loci regulated by histone modification [62]. Double-stranded DNA is wrapped around histone octamers [63] (Fig. 4). The third histone (H3) of the octamer complex has a protein tail that may be enzymatically modified, affecting the steric folding of the other histones and adjacent proteins. The most commonly modified residues of the histone tail are the 4th lysine (K4) on H3, which is regulated by methylation (e.g., H3K4me1) [64] and the 27th (H3K27) which can be mono, di, or tri-methylated at either promoters or enhancers and is almost universally associated with gene repression (Fig. 4). Loss of function mutations in chromatin-modifying genes are over-represented in the TCGA cohort including 10 of the top 39 genes [65] (Figs. 2, 4).

Chromatin modifiers with histone methyltransferase (KMT) activity enzymatically add methylation marks to lysine tails, and the MLL (mixed lineage leukemia) or KMT (lysine methyltransferase) family of methyltransferases are the most commonly affected in bladder cancer [66]. These genes include *KMT2D* (*MLL2* or *MLL4*), *KMT2C* (*MLL3*) and *KMT2A* (*MLL1*) mutated in 28, 18, and 11% of muscle-invasive bladder cancers [65]. Compared to other solid tumors, bladder cancer has the highest mutation rates of *KMT2D* and *KMT2C* among TCGA cancers [67]. Evaluation of tumor molecular subtypes suggests that loss of function mutations in *KMT2C* and *KMT2D* may occur more commonly in basal tumors in humans and BBN mice [68]. Histone acetyltransferases (HATs) enzymatically add acetyl groups to H3K27 at enhancer sites, resulting in transcriptional activation. The two most commonly mutated HATs in bladder cancer are *EP300* (15%) and *CREBBP* (12%) [65]. Loss of HAT function could cause increased enhancer silencing due to increased H3K27me3, favoring polycomb-mediated repression (see below) [69].

Enzymes that remove methyl groups from histones are "erasers." The most commonly mutated histone demethylase in bladder cancer is *KDM6A* (*UTX*) [65]. *KDM6A* was mutated in 26% of MIBC compared to 54% of non-invasive cancers, suggesting KDM6A loss may occur more commonly with early stage cancers or *RAS/FGFR3* signaling which are predominantly found in early stage cancers [53]. There may be a gender association with *KDM6A* loss, as females with non-invasive tumors had *KDM6A* mutations in 74% of cancers while men had mutations in only 35%.

The chromatin modifiers KMT2D, KMT2C, and KDM6A assemble with other proteins to form larger multi-protein complex called COMPASS (Complex of Proteins Associated with Set 1) [70]. Coordinating COMPASS function to simultaneously demethylate histones (KDM6A) at some loci (e.g., H3K27me3) and add methyl groups at other loci (e.g., H3K4me) with histone methyltransferase, COMPASS can efficiently alter the transcriptional landscape of a cell. Mutual exclusion of mutations in *KMT2D/KMT2C* and *KDM6A* in the first TCGA cohort suggested that while enzymatically distinct, KDM6A and KMT2D/C may co-operate to have similar function [8]. One hypothesis may be that mutation of KDM6A or KMT2C/D is sufficient to destabilize the entire COMPASS complex. The complementary activity of gene repression is mediated by the polycomb-repressor complex-2 (PRC2) [69], comprising the subunits EED, SUZ12 and the SET enzyme enhancer of zeste-2 (EZH2). EZH2 is the histone methyltransferase that binds H3K27 catalyzing H3K27me3 and resulting in chromatin condensation and gene repression at both promoter and enhancer sites [71]. While there are no significant mutations in *EZH2* in the TCGA cohort, increased expression of EZH2 is associated with invasive cancers, higher grade, epithelial–mesenchymal transition (EMT), and aggressiveness. Pharmacotherapeutic studies investigating the use of EZH2-inhbitors (EZH2i) have demonstrated that loss of KDM6A may increase the sensitivity of bladder cancers to EZH2i therapy [72].

The ATP-dependent activity of moving histones and nucleosomes during remodeling of chromatin structure is powered by the multi-protein SWI/SNF complex. The major enzymatic component of SWI/SNF is ARID1A, mutated in 25% of muscle-invasive bladder cancers [65]. Loss of function mutations in *ARID1A* were increased in luminal tumors (31%) compared to basal tumors (18%) [65]. In non-muscle-invasive bladder cancers, mutations of *ARID1A* have been associated with resistance to BCG therapy [73]. As a possible therapeutic target, loss of function mutations in *ARID1A* in clear cell ovarian cancers resulted in synthetic lethality when these tumors were treated with EZH2i [74]. While not explored yet in bladder cancer, the interaction of SWI/SNF and PRC2 may be another possible therapeutic target.

Chromatin-modifying enzymes play a pivotal, but complex role in shaping bladder tumor subtype and aggressiveness. While there is a greater understanding of gatekeeper and caretaker function in malignancy, the role of chromatin regulation will require further investigation. Targeting of chromatin-modifying enzymes, potentially by combination with chemotherapy or immunotherapy, may offer therapeutic potential for treatment of patients with bladder cancer.

Metabolomics

Metabolites are endogenous or exogenous small low-molecular weight downstream intermediate or end products of genes and proteins in a living organism. The composition of all metabolites generated by a system in a living organism (e.g., cell, organ, tissue) constitutes a metabolome.

Metabolomics is the identification and quantification of all (non-targeted metabolomics profiling) or specified metabolites (targeted metabolomics profiling) in a biological sample (e.g., blood, urine) under a specified condition or disease and identification of metabolic pathways and genes associated with the measured metabolites.

Metabolomics utilizes analytical chemistry techniques and advanced computational methods to characterize complex biochemical mixtures. The diversity of the applications of metabolomics arises from the fact that it can be used to analyze a wide range of biological complexes, including solids (tissues), liquids (bio fluids), and gases (breath). Over the past 10 years, in bladder cancer (BLCA) research, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are commonly employed for metabolomics applications.

BLCA has profound metabolic alterations, which play a central role in tumor progression. Metabolomics helps to understand relevant alterations in the impaired metabolic processes in BLCA through the identification of tumor-specific metabolic biomarkers with potential diagnostic, prognostic, or predictive value. Cigarette smoking is the most important risk factor for the development of BLCA and the duration of smoking has been shown to greatly affect the grade and stage of the BLCA. At least 60 tobacco smoke compounds, belonging to different classes of chemicals compounds, are known to induce cancer in either laboratory animals or humans. Smokers with BLCA have elevated levels of methylated metabolites, hexosamine biosynthetic pathway intermediates, acetylated metabolites, polycyclic aromatic hydrocarbons (PAH)/their aromatic counterparts, and hydroxylated derivatives and show the deregulation of DNA methylation, nicotine metabolism, glutathione metabolism, and nucleotide metabolism compared to non-smokers. BLCA smokers also exhibited higher DNA adduct formation and DNA damage that lead to more aggressive BLCA [75].

Urinary metabolomics analyses discriminate BLCA patients from healthy ones and show potential as an alternative or supplement diagnostic procedure to cystoscopy. Elevated levels of urinary nicotinuric acid and trehalose were identified in low-grade BLCA patients [76].

Currently, studies from tissues, serum, and urine BLCA patients show distinct signatures of altered metabolite levels that are indicative of specifically disrupted metabolic pathways including aromatic amino acid, glycolysis, citrate cycle, lipogenesis, nictotinamide, and xenobiotic metabolism. The trend in alteration of some metabolite levels is closely related to aggressive cancers, suggesting that these characteristic metabolites might play a vital role in the pathogenesis of BLCA. These results might also provide new candidates for invasive detection and surveillance of BLCA and could be exploited as potential biomarkers for non-invasive diagnosis and treatment of BLCA.

Immunotherapy and the immune landscape

Like melanoma and renal cell carcinoma, bladder cancer has long been considered to be responsive to immunotherapy. Frontline therapy for high-risk NMIBC involves thorough local resection, followed by intravesical therapy with Bacillus Calmette–Guérin (BCG), an attenuated form of *Mycobacterium bovis*, which prevents recurrence and progression and is an important adjunct to complete transurethral resection of all visible papillary disease [1]. Although the complex mechanisms that mediate the effects of BCG are still being elucidated, innate inflammatory cytokines (including death receptor ligands like TRAIL) [77] and T cell-adaptive immunity [78, 79] are both involved. Pre-clinical studies demonstrated that bladder cancer cells can internalize BCG via a fibronectin-dependent pathway [80], leading to release of a variety of different inflammatory cytokines that probably function as a "danger" signal to initiate the downstream immune response [81]. Aside from the cancer cells themselves, antigen-presenting cells also internalize BCG and carry it to draining lymph nodes, where a BCG-specific T cell response is initiated [79]; if tumor cells are somehow able to present BCG peptides themselves, BCG-specific T cell responses may contribute to tumor cell killing. Other cell types in the bladder also respond to BCG in ways that probably contribute to the anti-tumor response. For example, BCG stimulates neutrophils to display TRAIL [77], which can bind death receptors on tumor cells to initiate apoptosis. The relative contributions of various mechanisms to the anti-tumor effects of BCG are probably tumor dependent, and much more work needs to be done to understand this heterogeneity [81]. Perhaps most importantly, the role of tumor-specific neoantigens in the response to BCG is also still not clear and is the subject of active investigation.

Although BCG is initially effective in the vast majority of patients, a significant fraction present with or develop BCG-resistant disease. Also known as BCG-unresponsive disease [82], clinical studies have demonstrated that, stage for stage, this disease is more aggressive [1], leaving surgeons with the conundrum of whether or not to continue conservative therapy or proceed to radical cystectomy. Preliminary data have shown that BCG-unresponsive NMIBCs express higher levels immune checkpoint molecules [83], which have prompted the design of clinical trials to test the effects of systemic anti-PD-L1 antibodies in BCG-unresponsive disease, including SWOG's S1605 FDA registration trial. Parallel trials are evaluating the efficacy of other intravesical therapies. One of the more exciting approaches involves intravesical delivery of an adenoviral construct that induces sustained production of

interferon-α (IFNα) in the urothelium [84, 85]. The results of a large Phase II trial demonstrated that this drug, called Instiladrin, produced clinical responses in at least 30% of patients with BCG-unresponsive disease [85], and an FDA Phase III registration trial recently completed accrual. It may also be possible to augment the effects of BCG or Instiladrin by combining them with systemic checkpoint inhibitors, and clinical trials to test these approaches are either already open or planned for next year.

Clinical trials revealed that the clinical activity of anti-PD-L1 antibodies correlates with tumor mutational burden (TMB) [86], presumably because highly mutated tumors contain more neoantigens that can be recognized by T cells. Indeed, these agents recently received FDA approval in cancers with DNA mismatch repair deficiency [87], which results in very high TMBs, irrespective of the tissue of origin. In an early Phase II trial, the anti-PD-L1 antibody atezolizumab (Roche/Genentech) produced significant and sustained clinical responses in approximately 20% of patients [20], and since this observation was published, five different anti-PD-L1 antibodies have been approved for advanced MIBC. As is true in other cancers, clinical activity was higher in patients whose tumors have high TMBs [20]. Perhaps not surprisingly, responses were also linked to the presence of DDR mutations [88], which are associated with higher TMBs. Tumors with strong APOBEC signatures also have higher TMBs [14], so studies are underway to evaluate the power of APOBEC signatures to predict response.

Genomic studies have revealed that the molecular subtypes of bladder cancer are heterogeneous with respect to their content of various immune-related gene expression signatures and predicted T lymphocytes [89]. T cell infiltration (but also immunosuppression) was highest in the claudin-low basal tumors and lowest in the uroA/luminal papillary tumors. Analyses of RNAseq data from the completed clinical trials suggest that the molecular subtypes may be associated with response or resistance to immune checkpoint blockade [20, 21], but the RNAseq data are only now becoming available in the public domain, so these results must be reproduced. Working with Jaegil Kim and David Kwiatkowski from TCGA and Joshua Meeks from Northwestern, we have obtained preliminary evidence for a link between membership in the TCGA neuronal (neuroendocrine) subtype and survival in the patients treated on a trial (IMvigor210) with atezolizumab (manuscript submitted) and are anxious to seek validation of this observation in additional datasets.

Heterogeneity in peripheral immune status also appears to contribute to clinical outcomes in patients with NMIBC or MIBC. For example, neutrophil-to-lymphocyte ratios (NLRs) are associated with survival after radical cystectomy [90] and with disease recurrence and progression after TURBT [91]. A high NLR probably reflects a systemic imbalance between the innate and adaptive arms of the immune system symptomatic of a chronic inflammatory state. The NLR is very attractive as a cancer biomarker because it can be generated using routinely collected clinical data and therefore does not add any additional cost.

There also appears to be an interesting connection between the bacteria that colonize the gut (the gastrointestinal "microbiome") and the response to immune checkpoint blockade. Recent studies demonstrated that the efficacy of immune checkpoint blockade was associated with specific bacterial colonies in patients with several types of cancer, and antibiotic use was associated with lower response rates [92–95]. Importantly, fecal bacterial transfer studies in mice established a cause–effect relationship between these bacteria and response. Ongoing studies are exploring the relationship between the gut microbiome and response to BCG and the possibility that bacterial colonies in the bladder and other locations may also contribute to these outcomes.

Targeted therapy

Activating mutations and fusions targeting fibroblast growth factor receptor-3 (*FGFR3*) are present in the majority of NMIBCs [2] and a significant fraction of luminal papillary MIBCs [14] (Fig. 2). Preclinical studies demonstrated that cell proliferation in human bladder cancers that contain these mutations was blocked by specific FGFR inhibitors [96], prompting the evaluation of FGFR inhibitors in patients with bladder cancer. Unfortunately, the first ones evaluated had low potency and specificity and did not have significant clinical activity [97]. A more recent experience with the more potent and selective inhibitors, BGJ398 [98] and JNJ-42756493 (erdafitinib), has been much more positive—the former produced RECIST criteria response rates of 25% in heavily pretreated, marker-selected patients, and the activity of erdafitinib was even higher at 40% (Siefker-Radtke, personal communication). The promising clinical activity of erdafitinib prompted the FDA to grant the drug breakthrough status in March 2018, and a large international Phase 3 trial is underway. There is also strong interest in combining FGFR inhibitors with immunotherapy based on preliminary data, suggesting that the former might reverse the "immune desert" phenotype observed in luminal papillary cancers.

Muscle-invasive bladder cancers also contain several other attractive biological targets, including amplified EGFR [99] and mutated *ERBB2* and *ERBB3* [8, 14] (Fig. 2). In a recently completed clinical trial of the pan-ERBB inhibitor, afatinib, patients whose tumors contained activating *ERBB3* mutations or *ERBB2* amplification received clinical benefit, whereas none of the wild-type tumors were associated with response [100]. Whether or not ERBB antagonists will promote the effects of immunotherapy is an important

unanswered question. In addition, it will be important to determine how durable the responses are in patients treated with these agents.

Pre-clinical models

The acquisition of more detailed information about the heterogeneity of human bladder cancers has stimulated progress in the design and development of better pre-clinical models. However, the starting point for these efforts is encouraging. In spite of the neglect from basic research funding sources, bladder cancer investigators have established a relatively large number of conventional bladder cancer cell lines, and these models have functioned as the primary tools for research for decades. However, there is now concern that continued maintenance in tissue culture selects for in vitro growth and causes genomic and/or epigenetic changes that make the biological properties of established cell lines diverge from the original tumor. Recently, a large panel of 40 conventional human bladder cancer cell lines was subjected to whole exome sequencing and whole transcriptome mRNA expression profiling analyses [101]. Overall, the results of these analyses confirmed that the cell lines displayed more extensive genomic instability than typical cohorts of primary human tumors and also confirmed that the cell lines retained many important genomic characteristics. Also, many of the lines could be assigned reasonably well to the bladder cancer molecular subtypes.

A handful of rodent models have also been available to investigators for many years. One of the most popular models involves exposing rodents to nitrosamine 4-hydroxybutyl(butyl)nitrosamine (BBN) via their drinking water. This results in dose- and gender-dependent carcinogenesis that produces bladder cancers that progress from NMIBC to MIBC with fairly reliable kinetics. This model has been used to generate important insights into the molecular mechanisms of bladder cancer carcinogenesis, but recent studies have demonstrated that all of the muscle-invasive tumors that arise in these mice belong to the basal molecular subtype [99, 102], and the results of whole exome sequencing suggested that APOBEC-mediated mutagenesis does not play a significant role in their progression [103]. Therefore, the relevance of the BBN model may be limited to basal human cancers without APOBEC mutagenesis signatures.

Some genetically engineered mouse models have also been available for some time [104–106]. Most of them have relied on the use of the bladder-selective uroplakin (UPK) promoter to drive the expression of various oncogenes or to induce deletion of tumor suppressors, and we now know that uroplakin is a biomarker for luminal cancers. Indeed, a recent work has demonstrated that nearly all of the tumors that arose in mice in which both p53 and PTEN were deleted under the control of a uroplakin promoter ("UPPL" mice) belonged to the luminal subtype, and comparisons of BBN-induced and UPPL tumors demonstrated differential immune infiltration levels and responses to anti-PD1 antibodies, consistent with the patterns observed in patients [102]. However, all of these older mouse models were generated without much concern for making them high-fidelity copies of human cancers. Therefore, aggressive efforts are underway to improve them and to create new models to address these deficiencies.

Patient-derived xenograft (PDX) models may better represent human disease characteristics and heterogeneity than xenografts derived from conventional cell lines [107]. In this approach, pieces of fresh tumor are implanted directly into immunodeficient (usually NOD/SCID common cytokine γ-chain-deficient, otherwise known as "NSG") mice, thereby avoiding adaptation of tumor cells to tissue culture. Once established, these PDX models can be frozen and recovered for banking and transfer to other investigators. Genetic drift still occurs in these models, but it is probably less extensive than that observed in conventional cell lines.

An even newer approach involves culturing primary human tumor cells in three-dimensional spheres and specialized growth medium [108, 109]. These so-called organoids also appear to preserve key features of the parental primary tumor, and they can also be frozen and recovered and used to establish human tumor xenografts in immunodeficient mice. Importantly, NMIBCs grow well in organoid cultures [109], whereas it has been extremely challenging to establish conventional cell lines from these tumors. Analyses of genetic drift confirmed that the effects of organoid culture on the tumor genomic landscape was modest but still measureable, and most organoids derived from luminal tumors acquired a more basal molecular phenotype ex vivo [109]. Interestingly, the luminal phenotypes of these organoids were restored when they were used to establish orthotopic tumors in mice [109], suggesting an important role for the tumor microenvironment in maintaining the luminal phenotype.

These new models will probably be particularly valuable for studying the tumor–stromal interactions that control drug resistance and invasion and metastasis. To date, all of the work focused on the latter has employed xenograft models generated from established human cell lines. This work has implicated mechanisms related to "stemness", basal biomarkers, and EMT, and the results suggest that interactions between tumor cells and macrophages play important roles [110, 111]. A parallel work employing human breast cancer organoids has also implicated "basal" mechanisms that are activated in the tumor periphery in the invasion and metastasis of luminal tumors [112], where it appears that tumor–matrix interactions also play important roles. Therefore, it seems likely that bladder cancer organoids can be

used to exploit the published observations in breast cancer to determine whether similar mechanisms control invasion and metastasis in the bladder.

With this said, the extent to which tumor-immune interactions can be studied in immunodeficient mice is very limited. Recently, investigators have begun to establish humanized immune systems in NSG mice by implanting human hematopoietic stem cells [113]. Studies have shown that immune checkpoint inhibitors can promote T cell-mediated tumor recognition in these models, although the fact that the T cells and the tumor are from different people means that alloreactivity probably contributes significantly to the effects. There are also challenging differences in baseline immune function that probably make spontaneous and syngeneic mouse models better tools for immunology research.

Future directions

The molecular annotation of primary bladder cancers has identified a large number of candidate mechanisms that must be functionally validated. Most of the mutations and copy number variations that have been identified have unknown functional effects. Of particular importance will be the further characterization of mutations in chromatin-modifying genes, which are present in large percentages of NMIBCs and MIBCs. Some of these and other genes are mutated at different frequencies in basal and luminal cancers, so it will be interesting to study their biological effects in mouse models using promoters that selectively modulate them in different cell layers within the normal urothelium. There is also a great need to deepen our knowledge about the molecular characteristics of NMIBCs (particularly, CIS and high-grade T1 tumors) and the bladder cancer variants.

The exciting clinical activity of immune checkpoint blockade in bladder cancers makes generating more robust preclinical models for mechanistic studies an extremely high priority. This will probably be accomplished first by engineering spontaneous tumor models so that they contain the most relevant molecular features of human tumors; however, the development of better methods to "humanize" immunodeficient mice should be pursued in parallel. It will be important to consider the possible impact of the microbiome and other environmental factors when characterizing these models. Within this context, deeper characterizations of the gut, bladder, and other microbiomes and their relationships with responses to immunotherapy (BCG, immune checkpoint blockade, and others) must be carried out in primary patient samples.

Now that the molecular characteristics of the tumor cell compartment are growing clearer, more attention is needed to characterize the biological characteristics of the tumor-associated stromal cells, beyond current efforts with immune profiling. For example, the unique characteristics of the p53-like/infiltrated tumors may be controlled by tumor cell interactions with cancer-associated fibroblasts and specific extracellular matrix proteins. From work in other malignancies, it appears likely that we will encounter significant heterogeneity in different tumors. This work will be critical to our ability to identify the molecular mechanisms involved in progression from NMIBC to MIBC (associated with invasion) and metastasis that will provide additional candidate therapeutic targets for therapeutic intervention.

The biological insights resulting from these recent studies have significant near-term clinical implications. Retrospective studies indicate that the presence of DDR mutations and basal molecular subtype are associated with benefit from cisplatin-based neoadjuvant chemotherapy; if these observations are confirmed prospectively in the S1314 clinical trial, these biomarkers will enter clinical practice very soon. Similarly, pretreatment measurements of TMB and molecular subtype membership could soon inform the clinical utilization of immunotherapy with immune checkpoint blockade and/or BCG. Finally, the presence of activating mutations, fusions, or amplification involving *FGFR3*, *ERBB2*, or *ERBB3* could identify patients who would obtain benefit from clinically available small molecule inhibitors. After decades of modest progress, the future looks bright for the development of precision medicine for patients with bladder cancer.

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