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Advances in bladder cancer biology and therapy

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Abstract

The field of research in bladder cancer has seen significant advances in recent years. Next-generation sequencing has identified the genes most mutated in bladder cancer. This wealth of information allowed the definition of driver mutations, and identification of actionable therapeutic targets, as well as a clearer picture of patient prognosis and therapeutic direction. In a similar vein, our understanding of the cellular aspects of bladder cancer has grown. The identification of the cellular geography and the populations of different cell types and quantifications of normal and abnormal cell types in tumours provide a better prediction of therapeutic response. Non-invasive methods of diagnosis, including liquid biopsies, have seen major advances as well. These methods will likely find considerable utility in assessing minimal residual disease following treatment and for early-stage diagnosis. A significant therapeutic impact on patients with bladder cancer is found in the use of immune checkpoint inhibitor therapeutics. These therapeutics have been shown to cure some patients with bladder cancer and significantly decrease adverse events. These developments provide patients with better monitoring opportunities, unique therapeutic options and greater hope for prolonged survival.

Bladder cancer is among the most prevalent cancers worldwide, with 549,393 new cases reported in 2018 (REF.1). This disease can present as non-muscle-invasive bladder cancer (NMIBC), muscle-invasive bladder cancer (MIBC) or as a metastatic form of the disease, with each having different molecular drivers. While there were few advances in clinical

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management of bladder cancer over the past three decades, this trend has been considerably altered over the past several years. Advances in our understanding of bladder cancer biology, coupled with large-scale gene expression and sequencing efforts, have led to more clinically favourable targeted treatments and effective immunotherapies. These advances have also provided clinicians with the ability to characterize bladder cancer into distinct molecular and histological subtypes (FIG. 1).

Sequencing and gene expression studies have led to the discovery of numerous DNA, RNA and protein biomarkers of bladder cancer. This provides the opportunity for a tectonic shift in how bladder cancer is diagnosed and how recurrences are detected. The sequencing and gene expression studies have also shown that several distinct molecular signatures and subtypes exist, providing a more accurate prediction of disease progression and therapeutic response as will be discussed in this Review. Additionally, clinicians hope to supplant invasive cystoscopy with blood-based and urine-based diagnostics soon. Furthermore, recent successes in immunotherapeutic regimens and renewed interest in the tumour microenvironment (TME) have expanded the repertoire of potential targeted treatments[2]. These advances are shifting how we manage the patient with bladder cancer, with the expectation that increases in overall survival and improvement in quality of life will continue their upward trend[3]. In this Review we will examine recent developments in the molecular and translational aspects of bladder cancer biology and discuss their current or potential future clinical applications in the management of this disease. Prior reviews on animal models and molecular biology of bladder cancer will serve as background, and their contents not reiterated here.

Genetic alterations in bladder cancer

Next-generation sequencing helped provide the wealth of molecular information needed for molecular classification of tumours from patients with bladder cancer. Whole-transcriptome mRNA profiling of tumour tissue samples from patients allowed researchers to establish a molecular taxonomy for bladder cancer[4–10], while still recognizing there is some variation across molecular subtype classification due to patient selection, methodology differences[11–15] and pathological and clinical criteria[16]. Bladder cancer was found to be one of the most frequently mutated human cancers, following lung and skin cancer in mutation rates[17,18]. Chief among these mutations is mutations in the promoter of the gene encoding telomerase reverse transcriptase (TERT), occurring with a frequency of 70–80% in patients with bladder cancer[19–22]. The identification of frequent mutations has, in turn, promoted novel therapeutic approaches, as well as urine and blood surveillance for both early detection and disease monitoring following treatments.

Non-muscle-invasive molecular subtypes

In clinical practice, around 80% of patients with bladder cancer have a diagnosis of NMIBC, with good life expectancy (5-year survival rates greater than 85% (REF. 23)) and a low chance of developing invasive diseases[24,25]. However, despite advancements in research and care, the 5-year survival rate for NMIBC has not markedly changed, and the frequent recurrence of NMIBC has put a heavy load on public health systems[23,26,27]. Multiple

studies have characterized DNA and RNA alterations in NMIBC. Deletions in chromosome 9 (harbouring the *CDKN2A* gene) as well as mutations in genes encoding fibroblast growth factor receptor 3 (FGFR3) and PI3K and the promoter of the gene encoding TERT, were identified as early events in urothelial malignancy[28–31]. Hedegaard and colleagues performed a comprehensive transcriptional analysis including 460 patients with early-stage NMIBC (345 patients with stage Ta NMIBC, 112 patients with stage T1 NMIBC and 3 patients with carcinoma in situ) and 16 patients with MIBC and identified three major subclasses with basal-like and luminal-like characteristics and distinct clinical outcomes (UROMOL study)[32]. Class 1 tumours showed enrichment in early cell cycle genes and are associated with good prognosis. Class 2 tumours exhibited high expression of late cell cycle genes and are predominantly associated with high-risk NMIBC. Class 1 and class 2 tumours showed high expression of `uroplakins`, markers of bladder luminal/umbrella cells. Class 3 tumours were enriched in *KRT5* and *KRT15* expression, which are markers of undifferentiated or basal cells, as well as elevated expression levels of long non-coding RNA. Importantly, class 2 tumours are associated with lower rates of survival compared with class 1 or class 3 tumours. Furthermore, that 14 of 16 MIBCs were classified as class 2 tumours highlights the similarity between MIBC and high-risk NMIBC. Moreover, proteome analysis on tissue specimens from 117 patients (98 patients with NMIBC and 19 patients with MIBC) led to the identification of three NMIBC proteomic subtypes (NPS)[33]. NPS1 represented a small group (17%, 17/98) of mostly T1 grade 3 high-risk samples with overexpression of an immune and/or inflammatory profile, whereas NPS2 (43%, 42/98) showed a more immune infiltrated/mesenchymal phenotype. NPS3 (40%, 39/98) showed enrichment of luminal and differentiation markers KRT20, CDH1 and uroplakins. They also exhibit high expression of undifferentiation markers KRT5 and ITGA6/ITGB4, which resembles class 1 and class 3 tumours from the UROMOL study. In line with the clinicopathological feature, NPS3 patients were of the lowest risk group with better survival outcome. A major goal in the future will be to coalesce the genomic, transcriptomic and proteomic classifications into unified classifications and to increase the feasibility of gathering the needed data from each patient for clinicians to make the most informed choice.

Muscle-invasive molecular subtypes

Dissecting tumour molecular signatures and subtypes is also urgently needed for MIBC, in which the tumour has advanced beyond the epithelial layer and into the muscle (FIG. 1). To address this need, classification by molecular subtypes has been undertaken by several groups, published previously and summarized here. A great number of criteria are taken into consideration for these classifications (Supplementary Table 1). However, a consensus MIBC molecular classification was achieved with a single sample classifier integrating six non-overlapping patient subtypes (FIG. 1), which was also highly consistent with previous classification studies[34]. This consensus classification included luminal papillary (LumP), luminal non-specified (LumNS), luminal unstable (LumU), stroma-rich, basal/squamous (Ba/Sq) and neuroendocrine-like (NE-like) subtypes[34]. In the consensus classification, luminal tumours (LumP, LumNS and LumU) showed enriched urothelial differentiation, active *PPARG* and *GATA3* regulons and *FGFR3* genetic alterations (mutation, fusion or genomic amplification). The LumP subtype was the least aggressive subtype and was more often associated with lower stage (T2) and younger patients. This subtype

was frequently associated with *FGFR3* mutations and increased *FGFR3* regulon activity, suggesting a potential benefit from FGFR3 inhibitors. LumP tumours also harboured more frequent mutations of *KDM6A* and deletion of *CDKN2A*, yet harboured wild-type *TP53*. LumNS tumours were enriched with micropapillary histological variants and were associated with carcinoma in situ, a rare non-muscle-invasive type with a greater chance to evolve to muscle-invasive disease. LumNS tumours showed *PPARG* amplification, upregulation of *PPARG* downstream target genes, and *ELF3* mutations. LumU tumours exhibited a significant enrichment in genomic instability and copy number variations. They also exhibited increased mutations in the genes encoding the apolipoprotein B mRNA editing catalytic polypeptide-like family of proteins (APOBEC). Moreover, LumU tumours exhibited the highest levels of *TP53* and *ERCC2* mutations, the latter of which is associated with sensitivity to chemotherapeutic agents.

The stroma-rich subtype displayed intermediate levels of urothelial differentiation and is notable for prominent stromal cell infiltration and high levels of smooth muscle, endothelial, fibroblast and myofibroblast gene signatures. Stromal-rich tumours were enriched with T and B cell markers. Ba/Sq subtype tumours displayed high expression levels of basal cell marker genes *KRT14*, *KRT5* and *KRT6*, as well as loss of luminal cell marker genes *GATA3* and *FOXA1*. Tumours of the Ba/Sq group were aggressive with poor prognosis, and were often found in female patients and were of higher stage. In Ba/Sq tumours, the most frequently mutated genes were *TP53* and *RB1*. Regulon analysis showed that Ba/Sq tumours are enriched with *STAT3*, *HIF1A*, and *EGFR* regulon activities. Like the stroma-rich subtype, Ba/Sq tumours contained high levels of non-tumour cells. The NE-like subtype is the most homogenous subtype, with 81% of tumours displaying neuroendocrine histological features and evidence of immune infiltration. Moreover, *TP53* was ubiquitously mutated in NE-like tumours, co-occurring with *RB1* mutations and deletion. The NE-like subtype was also the most aggressive subtype in patients, resulting in only 1-year median overall survival.

To date, molecular subtyping of MIBC has mainly focused on stratifying global mRNA expression, which comprises less than 2% of total transcription, due to the majority of transcribed genes being ribosomal RNAs and non-coding RNAs. Robertson and colleagues performed tumour clustering based on the expression levels of non-coding RNAs, including long non-coding RNAs and microRNAs[7]. Long non-coding RNA clustering identified a tumour group with high-purity, papillary histology and organ-confined cancer7, which was also observed in a follow-up study[35]. Patients with tumours of this subtype showed enhanced activity in pathways involving FGFR3, Sonic hedgehog (SHH) and wild-type p53 proteins[7,35]. As genomic regions encoding mRNA represent only 1.1% of the human genome, with intronic (24%) and intergenic (75%) DNA[36] constituting the rest, genetic alterations in intronic and intergenic DNA regions are not uncommon. In bladder cancer, investigators have examined mutation patterns such as those associated with APOBEC editing featuring C>T and C>G mutations[37,38]. Among all cancer types, MIBC has the highest enrichment score of APOBEC-specific mutations[38]. The most famous APOBEC-specific mutations are in the *TERT* `gene promoter`, and bladder cancer has the highest frequency of mutations in this region[39] (see *TERT* promoter alterations).

Other non-coding mutation elements have been identified in bladder cancer, including mutations in other promoters (*PLEKHS1*, *TBC1D12*, *WDR74*, *LEPROTL* and *PLXDC1*), in 3′ untranslated regions (*TBC1D12*, *WDR74* and *LEPROTL1*) and in an enhancer for *ADGRG6* (REFs[40,41]). Molecular subtyping of bladder cancer is finding application in the clinical setting (BOX 1).

TERT promoter alterations

TERT promoter mutations are found in 70–80% of patients with urothelial bladder cancer, while it has been reported that the frequency of mutations in normal urothelium is very low[19–22,42–47]. TERT, the catalytic subunit of the telomerase holoenzyme, is responsible for preventing telomere shortening by adding sequences to chromosomal ends. Telomerase activity is high in embryonic and normal stem cells but nearly undetectable in most somatic cells due primarily to transcriptional downregulation of *TERT* expression[48]. *TERT* gene promoter mutations in urothelial cancer cell lines lead to higher levels of TERT expression and enzymatic activity compared with wild-type cell lines[49]. Compared with low TERT expression, high TERT expression is associated with reduced disease-specific survival of patients with bladder cancer[49]. TERT re-expression in cancer cells commonly occurs because of increased promoter activity caused by two hot-spot point mutations in the core promoter region at −124 C>T (C228T) or −146 C>T (C250T) from the start codon ATG[49]. The C228T and C250T mutations are present in 50–70% and 8–15% of patients with urothelial bladder cancer, respectively[19–22,42–47]. Both mutations are mutually exclusive from each other[50–52], and both NMIBC and MIBC have these mutations at similar frequency[22,46,53]. Among various histological subtypes of urothelial bladder cancer, *TERT* promoter mutations have been reported in plasmacytoid variant[54], nested variant[55], papillary urothelial carcinomas[56] and glandular lesions[57]. Two studies examined the mutation status in inverted papillomas, with one reporting mutations in only 15% of cases[44] while the other reported no mutations[56]. Besides urothelial bladder cancer, other bladder cancer types have *TERT* promoter mutations. In bladder squamous cell carcinoma 80% of tumours have mutations[58]. In 11 patients with small cell carcinoma of urinary bladder that were examined, all had the C228T mutation[59]. Finally, in bladder adenocarcinoma, *TERT* promoter mutations were detected in 13% (2/15) of cases[60]. Thus, *TERT* promoter mutations are ubiquitous in bladder cancer types and are limited to the two specific mutations mentioned above.

Epigenetic modifications

Bladder cancer also exhibits significant epigenetic dysregulation such as changes in DNA methylation[61]. The Cancer Genome Atlas (TCGA) analysis of MIBC samples from patients has identified clusters of DNA hypomethylation and hypermethylation7. DNA hypomethylation appears to be more common in stage 1 and 2 MIBC and NMIBC than in MIBC of higher stages, while DNA hypermethylation is linked to silencing of tumour suppressor genes (for example, *TP53*, *RB1*, *CDKN2A* and *CDH1*) and is associated with aggressive disease7. In line with this, classification of MIBC according to a DNA methylation signature may provide a benefit for identifying predictive biomarkers and also treatment therapies[61].

Advances in tumour genomic analysis have also revealed that bladder cancer has a much higher mutational load than most other cancers, particularly in chromatin remodelling genes encoding proteins such as histone demethylase KDM6A, histone methyltransferases (KMT2C and KMT2D), histone acetylases (CREBBP and EP300) and the SWI/SNF chromatin remodelling complex (ARID1A)[7] (FIG. 2). Most of the alterations are inactivating mutations or deletions[7], indicating that loss of epigenetic regulation may be a major force in shaping bladder cancer progression. KDM6A is an X-linked histone demethylase specific for histone H3 Lys27 dimethylation and trimethylation[62], and research showed that its functional loss is a strong driver of bladder cancer progression. Silencing of KDM6A with short hairpin RNA promoted bladder cancer cell proliferation and migration in vitro, and xenograft tumour growth in vivo compared with controls[42]. Furthermore, conditional knockout of *Kdm6a* in the urothelium increased the risk of bladder cancer progression in female mice[63]. Consistent with this, the presence of *KDM6A* mutations in female patients with cancer predicts a poorer prognosis, in line with other sex differences observed in patients and discussed in the section Biological sex-based features[63]. Mice with *Kdm6a* deficiency and *Trp53* haploinsufficiency spontaneously developed carcinoma in situ in the urothelium and had accelerated disease when exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine treatment[64]. However, genomic sequencing has also revealed that deletion of *KDM6A* in bladder cancer cells led to upregulation of PRC2-mediated and EZH2-mediated transcriptional repression, which rendered these cells more sensitive to EZH2 inhibitors[65].

Together with KDM6A, the histone methyltransferases KMT2D and KMT2C participate in scaffolding nuclear regulatory structures called ‘`complex of proteins associated with set1`’ (COMPASS)[66]. Silencing *KMT2D* using small interfering RNA promoted bladder cancer cell proliferation and invasion in an in vitro transwell assay, as well as xenograft tumour growth in vivo, suggesting the gene has a tumour suppressor role in bladder cancer. Silencing *KMT2C* decreased histone H3 Lys4 methylation at enhancer regions, leading to decreased expression of DNA damage response genes (for example, *ATM*, *ATR*, *CHEK2* and *BRCA1*) and an increase in chromatin instability of bladder cancer cells.

CREBBP and EP300 are two closely related proteins whose histone acetyltransferase domain acetylate histones, thereby remodelling chromatin into different transcription states[67]. One-third of patients with bladder cancer harbour genetic alterations in the genes encoding EP300 and/or CREBBP, and it has been discovered that loss of histone acetyltransferase domain activity through mutations or deletions is associated with lower rates of survival of patients[68]. *ARID1A* is another frequently mutated gene in bladder cancer tumours7. ARID1A and the associated SWI/SNF complex are recruited to DNA double-strand breaks to facilitate the DNA damage repair[69]. Clinically, meta-analysis showed that higher levels of microsatellite instability and tumour mutation burden were found in patients with bladder cancer with genetic alterations of *ARID1A*, and this was associated with greater progression-free survival rates after immunotherapy[70] — showing that in addition to genetic and epigenetic modifications in bladder cancer cells, cancer cell-extrinsic factors and the TME are important to consider.

Tumour microenvironment

The TME consists of malignant and non-malignant cells where cancer cells have co-opted non-malignant cells, including immune and stromal cells, into ‘helping’ them prosper (FIG. 3). It is increasingly evident that the interplay of these cellular compartments affects cancer progression and response to therapeutics. Therefore, current preclinical models incorporate these compartments and enable investigation of specific cell type-dependent contributions to cancer progression (TABLE 1).

Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are the predominant stromal cell type observed in the TME and have been implicated in the pathogenesis of multiple cancers[71]. While no defined marker profile has been established, these activated fibroblasts are often identified by expression of vimentin, α-smooth muscle actin, fibroblast activation protein (FAP), S100 calcium-binding protein A4 (S100A4) and platelet-derived growth factor receptor-β (PDGFRβ). While known for their role in remodelling of the extracellular matrix, CAFs have attracted attention as a potential therapeutic target due to their role in modulating the immune system and crosstalk with cancer cells. However, this concept is controversial as conflicting studies show that their function can be tumour promoting[72] and/or tumour inhibiting even in the same cancer type (in this case, pancreatic cancer), dependent on the setting[73]. This can be partially explained by the heterogeneity of CAF populations observed in the TME[74] and the lack of known mechanisms that regulate stromal differentiation during cancer progression. Immunohistochemical analyses of primary tumours from patients with bladder cancer have shown increased presence of CAFs compared with normal urinary bladder tissue[75], and expression of CAF markers CD90, FAP and PDGFRβ positively correlated with the aggressiveness of bladder cancer[76]. Additionally, a hierarchical cluster analysis of 344 patients with bladder cancer revealed a patient cluster with dominant expression of FAP to have a lower 5-year survival outcome[76]. These findings were further substantiated by a study that found expression of FAP positively correlated with muscle invasiveness and negatively correlated with survival in patients with bladder cancer of the basal phenotype[77]. In addition, stromal expression of CAF markers has been strongly correlated with expression of epithelial–mesenchymal transition (EMT) markers in cancer cells, and stimulated fibroblasts can induce cancer cell EMT in vitro[78]. Moreover, human CAFs promote resistance to cisplatin by increasing the antiapoptotic gene *BCL2* in cancer cells through IGF1-oestrogen receptor-α (ERα) signalling in vivo[79]. These studies indicate that CAFs play a significant role in bladder cancer progression and contribute to aggressive disease.

Tumour-infiltrating immune cells

The approval of `immune checkpoint inhibitors` (ICIs) for the treatment of metastatic bladder cancer (see Immunotherapies) has reinvigorated interest in the immune component of the TME for its prognostic and therapeutic potential. ICIs target negative regulating cell receptors on immune cells, predominantly T cells, and prevent those receptors from signalling, leading to reactivation of those cells and promotion of a durable antitumour response. Currently approved ICIs target cytotoxic T lymphocyte-associated protein 4

(CTLA4) and the PD1–PDL1 axis. However, there are significant research efforts focused on expanding the known repertoire of targetable co-stimulatory molecules[80].

Tumour-infiltrating lymphocytes.—Tumour-infiltrating lymphocytes (TILs) are indicative of a cell-mediated host response to tumour cells. Cytotoxic CD8+ T cells are well characterized due to their direct role in restraining tumour growth. On recognition of foreign antigens, CD8+ T cells can induce apoptosis through release of cytotoxins or cell-surface expression of death ligands[81]. In MIBC, quantification of TILs that locate to the tumour-adjacent stroma has been shown to predict immune phenotype, patient survival and molecular tumour subtype[82]. Tumours have been often classified by the extent of TIL presence, resulting in three classifications: tumours that are uninflamed with low infiltration of TILs, tumours that are inflamed with low or high infiltration of TILs, and tumours with moderate TIL infiltration yet lack PDL1+ immune cells[82]. Inflamed tumour phenotypes were also characterized by a chemokine gene expression signature. High inflammation in tumours was shown to be correlated with increased survival of patients with nearly any type of cancer. In bladder cancer, total tumour gene expression analysis has shown that an EMT-related gene expression profile correlated with increased T cell infiltration[83]. However, Wang et al. found that stromal cells contributed more to EMT-related gene expression than cancer cells. Their findings support the notion that the high levels of T cells observed in tumours are actually present in the stroma and are not directly interacting with cancer cells, rendering those T cells ineffective in their duties as antitumour agents. Consistent with this, patients with high CD8+ T cell infiltration and a low stroma-related gene expression signature have longer overall survival, while a high stroma-related gene expression signature is associated with worse outcomes in response to immune checkpoint blockade[83]. In the immune exclusion phenotype, which is common in metastatic urothelial cancer, CD8+ T cells are observed only in the peritumoural stroma[83], strongly supporting the notion that the advanced disease may have reduced T cell infiltration due to stroma-induced changes in the TME. These modulations include sequestration of T cells in stroma due to cytokine expression[84] and physical exclusion of T cells from the tumour bulk due to increased deposition of fibronectin and collagen fibres in the extracellular matrix[85]. Studies exploring tumour oncogenic pathways have identified *FGFR3* mutations primarily in non-inflamed tumours and β-catenin and peroxisome proliferator-activated receptor-γ as possible regulators of T cell exclusion[86]. From a therapeutic standpoint, reducing stromal cell influence by abrogation of transforming growth factor-β signalling, facilitated T cell infiltration and enhanced immune checkpoint therapy effectiveness[84].

Tumour-associated macrophages.—Macrophages residing in the tumour tissue or those that are recruited from the bone marrow to intratumoural regions of hypoxia and necrosis can become tumour-associated macrophages (TAMs)[87]. These TAMs potentiate cancer growth[88], metastasis[89] and resistance to therapy[90] through secretion of growth factors, cytokines and proteases. TAMs also promote chronic inflammation, a suppressing event for immune-related tumour cytotoxicity, through release of mediators such as tumour necrosis factor (TNF), transforming growth factor-β, IL-6 and IL-12 (REF.91). Although macrophages can be polarized across a continuum of phenotypes[92], they are generally subtyped as ‘M1’ or ‘M2’. The M1 lineage promotes acute inflammation in response to

pathogens and tumour cells, while the M2 lineage promotes chronic inflammation, which in turn leads to immunosuppression and protumour growth[93]. TAMs isolated from patients with bladder cancer are predominantly of the M2 type and are found in greater quantities in higher-grade disease compared with low-grade disease[88].

In patients with bladder cancer in situ, the disease has not invaded physiological boundaries yet (FIG. 1) and can be treated via intravesical instillations of a therapeutic agent, most commonly bacillus Calmette–Guérin (BCG). How BCG exerts its effect on bladder cancer has not been fully elucidated, but it stimulates a local immune response in the lumen of the bladder, with a collateral outcome being an enhanced immune response against the bladder cancer cells. First given to patients in 1977, this is an early example of immunotherapy. When the disease recurs after BCG treatment, it was found that it was in instances of high TAM infiltration, 47.6% versus 4.8% (REF.94). In MIBC, the frequency of galectin 9-positive (Gal-9+) TAMs positively correlated with tumour stage and grade[95]. Patients with MIBC and higher percentages of Gal-9+ TAMs had strong correlations with poorer prognosis, reduced intratumoural presence of CD8+ T cells and dendritic cells, and increased presence of immunosuppressive regulatory T cells (T_{reg} cells) and mast cells as compared with patients with lower percentages of Gal-9+ TAMs[95]. It was these patients who derived greater survival benefit following adjuvant chemotherapy[95]. Another subclassification of M2 macrophages, dendritic cell-specific C-type lectin (DC-SIGN) TAMs, have been observed in MIBC and are associated with poorer prognosis. Patients with MIBC and low levels of DC-SIGN TAM infiltration had more favourable survival outcomes after adjuvant chemotherapy than patients with high DC-SIGN TAM infiltration[96]. The latter group of patients exhibited increased levels of T_{reg} cells as well as increased levels of intratumoural cytotoxic CD8+ T cells, but the cells had reduced levels of the cytotoxins perforin, granzyme B and interferon-γ (IFNγ), while also showing increased expression of co-inhibitory receptors TIGIT and LAG3 (REF.96), indicative of an overall immunosuppressive environment in patients with high DC-SIGN TAM infiltration.

Other immune cells.—Stromal cells can recruit M2 macro-phages to the TME and promote T_{reg} cell infiltration[97]. Cancer cells themselves can recruit immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs), an umbrella term encompassing a wide range of immature myeloid cells, and defective dendritic cells that promote T cell tolerance of tumour antigens[98]. In normal tissues, myeloid cells promote a tolerogenic microenvironment to restrict excessive inflammation and prevent autoimmune disease[99]. In cancer, myeloid cells foster immune evasion, stimulate proliferation and promote invasion and metastasis[100], ultimately hindering immunotherapy. T_{reg} cells secrete inhibitory cytokines, granzyme A and granzyme B to suppress T cell activation and proliferation, as well as modulate dendritic cell maturation and function[101]. Urinalysis of patients with NMIBC revealed conventional T_{reg} cells are not substantially present before BCG instillation, but the treatment can induce a subset of PDL1+ T_{reg} cells that can persist after treatment102. With both subsets taken into account, high levels of urine T_{reg} cells were correlated with rapid recurrence following BCG therapy102. These findings support combining use of an ICI with BCG instillation for the treatment of NMIBC (see “Immunotherapies”).

In patients with bladder cancer, monocytic MDSCs (M-MDSCs) are enriched in cancerous tissues and suppress CD8+ T cell activities. A preclinical model indicated that gemcitabine treatment can induce bladder cancer cells to secrete C-C motif chemokine ligand 2 (CCL2), promoting M-MDSC recruitment and limiting response to therapy[103]. Inhibiting the CCL2 chemokine receptor alone blocked metastasis in models[104], and when combined with gemcitabine can reduce M-MDSC chemotaxis and increase overall survival in syngeneic mouse models of bladder cancer[103]. Additionally, targeting MDSCs with α-Ly6C and α-Gr1 antibodies in combination with immune checkpoint therapy was shown to reduce tumour volume and increase infiltration of CD8+ T cells in a murine xenograft model of bladder cancer[105].

Dendritic cells are antigen-presenting cells that recognize pathogen-associated and danger-associated signals to prime naive T cells and contribute to the antitumour immune response. Their dysfunction in the TME results in ineffective antigen presentation and T cell activation[100]. In NMIBC, high levels of tumourinfiltrating dendritic cells are associated with a poor response to BCG instillation[106]. Consistent with these findings, the ratio of CD8+ T cells to various immunosuppressive cell types has emerged as a prognostic marker in bladder cancer, as in many other cancer types. In NMIBC, a CD8+ T cell to MDSC ratio of less than 1 is predictive of shorter recurrence-free survival[107]. In MIBC, the CD8+ T cell to T_{reg} cell ratio is associated with response to neoadjuvant chemotherapy, with those having a ratio of less than 1 having no response[108].

While the TME field is fairly nascent in bladder cancer, advances in imaging and single-cell analysis technologies can further elucidate the significance of stromal and infiltrating immune cell types in cancer progression and therapeutic interventions[109]. Application of these technologies to clinical trials is especially appealing and may reveal mechanisms of therapeutic sensitivity and resistance. More broadly, single-cell RNA sequencing can be applied to survey stromal and immune cell populations, in conjunction with tumour cells, to determine favourable courses of treatment[110]. Applied to specific populations, such as T cells, single-cell RNA sequencing can identify tumour-specific states of CD8+ T cells and produce a gene signature predictive of clinical response to therapies such as ICIs[111]. The incorporation of TME components in patient care has the potential to significantly improve the diagnoses and treatment of patients with bladder cancer. While much remains to be discovered, current research indicates the findings will be well worth the investment.

Population-based features

Ethnicity and socio-economic factors

While patient care has greatly improved over the decades, there still exists a noticeable racial disparity in overall survival of patients with bladder cancer. In an analysis of age at diagnosis, disease stage and other variables between 1973 and 2014 with use of the SEER (Surveillance, Epidemiology, and End Results) database, racial differences were noted in bladder cancer[112]. In the American military health system, which provides universal health care to all beneficiaries, Black patients were more likely to present with MIBC than white patients[113]. However, white and Black patients were not significantly different in overall and recurrence-free survival regardless of muscle invasion. This suggests the racial disparity

observed in higher-grade bladder cancer may be partially due to societal factors affecting access to health care. Additionally, Black patients have lower overall survival than white patients following radical cystectomy for transitional cell carcinoma[114]. When considered in the context that Black patients are less likely to receive surgical treatment, and those that do receive cystectomies are in low-volume hospitals, the racial divide observed in bladder cancer is further deepened[114,115]. While biological factors contributing to advanced bladder cancer should not be discounted, socio-economic differences likely exist in the care that the two patient populations are receiving. Further studies are overdue to increase our understanding of this discrepancy.

Biological sex-based features

Patients with a female genitourinary system and hormonal environment are more likely to present with local advanced disease at the time of diagnosis even when significant covariates such as age and health-care access are considered[116]. Compared with patients with a male genitourinary system and hormonal environment, these patients may have a higher risk of progression, disease recurrence and death[117] when all risk variables are considered, but this is still a matter of significant debate. In some studies, reduced survival in these patients is maintained when that data are adjusted for age, disease stage, co-morbidity burden, socio-demographic factors and clinical management[118], driving hypotheses that differential exposure to environmental hazards, sex steroid hormone regulation and molecular variances may account for the discrepancies in incidence and mortality. Additional evidence for a role of sex steroid hormones in bladder cancer progression includes the observation that there is an increased risk of bladder cancer progression among postmenopausal patients[119] and reduced risk among patients who have experienced hormone replacement therapy[120]. While few studies have explored the contribution of the urinary microbiota to bladder cancer pathogenesis (see BOX 2), the male and female genitourinary flora are dominated by different genera that may partially account for the sex disparity[121,122].

In preclinical models, sex chromosomes and hormones have roles in bladder cancer development[63]. X-linked genes, such as *KDM6A*, can be sexually dimorphic and protect against bladder cancer. Androgen receptor (AR), ERα and ERβ are present in bladder tumours, albeit at low levels, and their expression patterns are similar between biologically male and female patients. However, specific percentages differ greatly between disease-type cohorts. In one comprehensive immunohistochemical study of 188 bladder tumours, 141 non-neoplastic bladders and 14 lymph node metastasis tissues, AR was found to be expressed moderately (42% of primary tumours and 71% of metastatic tumours), ERα was expressed infrequently (27% of primary tumours and 64% of metastatic tumours) and ERβ was expressed moderately (49% of primary tumours and 71% of metastatic tumours). All three receptors were more frequently observed to be expressed in lymph node metastases as compared with primary tumour, while expression of AR and ERα inversely correlated and expression of ERβ directly correlated with higher-grade disease[123]. Experiments in a carcinogen-induced mouse model have also demonstrated AR expression is crucial to bladder cancer initiation and progression[124], whereas ERα expression restrains initiation and growth[125] and ERβ expression supports growth and invasion[126]. These findings support the idea that ERβ is the prevalent ER observed to play a role in bladder cancer.

Retrospective studies of patients with prostate cancer treated with androgen deprivation therapy and patients with benign prostatic hyperplasia treated with 5α-reductase inhibitors revealed lower risk of development[127] and recurrence of bladder cancer[127–129] as compared with surgical treatment and radiotherapy or no ADT. The role of AR signalling in mechanisms pertinent to bladder cancer progression have recently been extensively detailed and will not be reiterated here[130]. Preclinical models have indicated AR-negative bladder cancer cells are more sensitive to ionizing radiation as compared with AR-positive cells[131]. Administration of the antiandrogen drug hydroxyflutamide enhanced radio-sensitivity, suggesting abolishing AR signalling may increase the efficacy of radiotherapy[131]. Taken together, the findings suggest AR is as a promising therapeutic target to reduce bladder cancer development and increase the efficacy of current treatments.

Molecular diagnosis

The current standard of care for bladder cancer diagnosis is cystoscopy and urine cytology[132] (TABLE 2). The advantages and disadvantages of cystoscopy from a patient and health-care system perspective have been well described. Urine cytology provides a unique opportunity to assess eluent from an organ and is non-invasive. However, it has lacked adequate sensitivity for low-grade bladder cancer[133,134]. Among bladder cancer diagnoses, nearly 25% will be given the aggressively growing muscle-invasive or metastatic classification[3]. Thus, developing highly accurate, cost-effective, non-invasive tests for bladder cancer diagnosis and surveillance is an area of need in clinical diagnosis, particularly for early and low-grade urothelial tumours[132].

Evaluation of RNA and DNA in urine from patients has been investigated for decades[135] as a diagnostic marker, yet has only recently been approved[136]. Such evaluations now include panels rather than one marker, along with various assessments such as measuring gene expression levels, sequence variations, histone modifications and DNA methylation. Recent advances in next-generation sequencing and isolation techniques have also shed light on the diagnostic value of cell-free DNA (cfDNA) detection and profiling[137,138]. A recent study showed importantly that, compared with urinary cellular DNA, urinary cfDNA more faithfully correlated with the genetic alterations in tumour tissue[139]. Of the mutations detected via circulating cfDNA in bladder cancer to date, *TERT* promoter mutations appear most commonly, making them perhaps the most useful for primary and secondary (recurrence) detection of bladder cancer[22,43,140–147]. *FGFR3* is also highly mutated in bladder cancer, and its use has been shown to offer excellent tumour detection sensitivity[22,43,140–146]. Panels incorporating *TERT* promoter mutations enable the diagnosis of bladder cancer months to years before clinical manifestation or positive cytology findings[141–143,145]. One recent study reported detection of *TERT* promoter mutations in urine 10 years before the presence of bladder cancer symptoms[148].

Like cfDNA in the urine, increased levels of cfDNA have also been detected in the plasma of patients when compared with levels from normal controls[149,150]. While most cfDNA is believed to come from normal haematopoietic breakdown[151], there are significant levels that come from tumours[150,152]. Thus, plasma cfDNA for early detection of disease and disease relapse is being rigorously studied in human cancers, including bladder cancer[153].

Assaying plasma cfDNA has been shown to non-invasively detect bladder cancer up to 4 years before the patient presented with the disease[154]. For example, in patients with NMIBC compared with patients who have been declared disease-free of bladder cancer, higher levels of plasma and urine cfDNA are detected[155]. Furthermore, as expected, the levels of plasma and urine cfDNA significantly increase on metastatic relapse[156], and analysis of serially collected plasma samples from patients led to identification of disease relapse earlier than by radiographic imaging after radical cystectomy[156–158]. Thus, the minimally invasive approach of isolating cfDNA from blood and urine is an extremely promising method of early detection and post-treatment monitoring of disease. The current limitations of identifying the cancer mutation signature in cfDNA above the normal background signal will diminish further with advances in next-generation sequencing and more detailed molecular subtyping.

Therapeutic advances

Non-muscle-invasive disease

Increasing intravesical dwell time and uptake of current therapeutic agents.— Relative to treating MIBC, treating NMIBC has a major advantage in that non-systemic chemotherapy can be applied into just the lumen of the bladder (intravesical chemotherapy), sparing the normal tissues in the rest of the body from the toxicity of the agents used. Furthermore, intravesical chemotherapy is quite effective, along with transurethral resection of the bladder, in reducing recurrence of disease[159,160]. Mitomycin C, epirubicin, thiotepa, gemcitabine and doxorubicin are the most commonly used agents for this purpose, and although they are effective, there is room for improvement. Thus, a number of clinical trials are ongoing to validate changes to the delivery of therapeutics (TABLE 3) in an intravesical setting. To increase the effectiveness, investigators have sought to increase the dwell time and thus contact time of therapeutics with regard to the tumour cells lining the bladder lumen through implantation of carriers that allow localized diffusion of the drug over time. This approach has been approved by the FDA for a number of eye treatments161. Several trials are using a gemcitabine-releasing intravesical system (TAR-200) in a 21-day dwell period in combination with nivolumab for treatment of NMIBC[162] and in patients with MIBC who are unfit for radical cystectomy[163]. Mixing chemotherapy agents in hydrogels is another approach to achieving sustained release and increasing the dwell time. A phase II, dose-escalation study investigated the efficacy and disease recurrence rate in patients with NMIBC treated with mitomycin C in hydrogel before transurethral resection of the bladder[164], with a follow-up phase IIb study[165] currently active. This approach was so successful in combating upper urinary tract urothelial cancer that it recently gained FDA approval[166].

There are also approaches to increase uptake of chemotherapy by cancer cells following intravesical administration. These include albumin-bound nanoparticles to deliver paclitaxel[167] or rapamycin[168] in bladder cancer. Preclinical studies showed that the delivery of paclitaxel wrapped in polymers reduced bladder weight and led to the paclitaxel concentration in bladder tumours being higher than that in cremaphor– paclitaxal-treated mice[169]. Another advance is the use of `photodynamic therapy` in clinical trials with promising results. In 2018, a phase Ib clinical trial of the photo-sensitizer TLD1433 was

completed on six patients with bladder cancer[170]. The therapy was well tolerated, and patients exhibited no evidence of disease at 6 months[171]. A phase II trial is currently recruiting to further explore the therapeutic benefits of this photosensitive molecule[172].

Immunotherapies.—The standard of care for patients with NMIBC with a high risk of recurrence is currently intravesical administration of BCG. This therapy reduces recurrence[171] and progression[173] and increases survival of high-risk patients[174], and can also be given as a maintenance therapy for patients at intermediate risk and high risk of NMIBC[175]. However, management of the 'BCG-refractory' patient[176] is the greatest challenge in the field as the lifestyle-altering radical cystectomy is currently the most common option. Therefore, combining BCG with other therapies is being evaluated, including combining BCG with IFNα[177], recombinant adenovirus IFNα[178], or a combination of IFNα, IL-2 and subcutaneous granulocyte–macrophage colony-stimulating factor (GM-CSF)[179]. The most exciting approach recently has been to investigate ICIs[180]. The FDA recently approved the ICI pembrolizumab, a PD1-blocking antibody, for administration to patients with BCG-refractory NMIBC or those unable or unwilling to undergo cystectomy[181], and 46% of the responding patients had a complete response lasting at least 12 months[182]. Currently, there are 35 phase II and phase III clinical trials assessing BCG in bladder cancer, and 148 active clinical trials assessing ICIs in bladder cancer, with some using a variety of BCG courses.

There are other immune-related approaches to targeting NMIBC in clinical trials. Similar in concept to BCG, there are viral particles that are administered intravesically that selectively infect cancer cells to elicit an immune response or deliver a unique cargo. These include an attenuated version of the measles virus[183], delivering an enzyme to locally activate chemotherapy[184], a virus to promote expression of IFNα specifically in cancer cells[185], the use of a coxsackievirus to selectively attack cancer cells and promote lysis, and an attenuated strain of Salmonella *enterica* subsp. *enterica* serovar Typhi (aetiological agent of typhoid)[186]. Additional therapeutic approaches for NMIBC and intravesical administration include a recombinant protein fusion[187], an IL-15 superagonist[188] and a recombinant plasmid that promotes diphtheria toxin expression preferentially in malignant cells[189], although this latter study was ended due to poor efficacy. Taken together, the number and variety of immunotherapies illustrate the current focus towards harnessing a patient's immune system to combat bladder cancer.

Muscle-invasive and metastatic disease

Molecular markers.—The main goals in the setting of MIBC are prevention of local and metastatic recurrence or treat such events successfully if they occur[190]. The current standard of care for muscle-invasive disease is neoadjuvant platinum-based chemotherapy followed by radical cystectomy[3,191]. One of the needs in this area has been finding predictors of chemotherapy sensitivity. Identifying patients who are chemosensitive would not only provide a benefit to them but would spare unnecessary toxicity and a possibly fatal delay in radical cystectomy for patients who are chemotherapy resistant[192,193]. A number of genes have been implicated in this process to various degrees. Copper transporter 1 (CTR1) transports cisplatin into bladder cancer cells[194], and patients with high expression

of *CTR1* in their tumours have more favourable outcomes than patients with low or undetectable tumoural *CTR1* expression[195]. Many chemotherapy agents generally function by binding to and damaging DNA. In bladder cancer, the decreased expression or loss of function of the DNA damage response genes *ERCC1* and *ERCC2* (REF. 196) and *BRCA1* (REF. 197) is a strong indicator of cisplatin sensitivity and increased patient survival[198,199]. Recently, a phase II clinical trial[200] has been testing a novel biomarker concept aimed at matching patient genomic profiles with a specific targeted therapy. This concept, coexpression extrapolation (COXEN), aims to predict patient tumour response on the basis of sensitivities of laboratory cell lines and their known genomic profiles, and has been the first such idea to be tested in a clinical trial[201]. The results of this phase II trial presented at ASCO 2019 annual meeting showed the COXEN Gemcitabine+Cisplatin score to be a significant predictor for downstaging when participants in the Gembitabine+Cisplatin and dose dense MVAC (Methotrexate+Vinblastine Sulfate+Doxyrubicin Hydrochloride (Adriamycin)+Cisplatin) arms were combined[202].

Host genetics are also important in chemotherapy response. In an analysis of 80 SNPs implicated in developing bladder cancer or response to platinum chemotherapy, six SNPs affecting *IL1B*, *CCND1*, *PARD6B*, *GALNTL4* and *XPA* have been associated with complete or partial response to neoadjuvant chemotherapy[203]. Of particular interest, gene expression of *ERAP2* has been linked to the genotype of loci rs2927608 and is inversely correlated with overall survival of patients with bladder cancer of the luminal subtype receiving immune checkpoint therapy[204]. In addition, one of the most common targets in bladder cancer is members of the FGFR family. In bladder cancer, expression of one or more of these family members is often elevated or activated by virtue of amplifications, activating mutations and/or gene fusions[205]. FGFR inhibitors are becoming a significant line of therapy for patients with MIBC, owing to increased molecular profiling that leads to the identification of patients with FGFR genetic alterations. In 2019 the first FGFR kinase inhibitor was approved by the FDA, erdafitinib, as a treatment for patients with bladder cancer who have an FGFR genetic alteration[206]. There are currently three additional clinical trials recruiting particpants to determine additional indications and regimens for erdafitinib. Infigratinib (BGJ398) is another FGFR inhibitor that has shown success in patients with MIBC who have molecularly profiled genetic alterations in FGFR genes. This drug has shown success[207] in an early-stage clinical trial[208], and in early 2020 a phase III clinical trial[209] was initiated to expand the investigation.

Another molecular directed approach involves the identification of tumours that express high levels of a specific cell membrane protein so that those tumours specifically can be treated with antibodies to those proteins. Linking a toxin to these antibodies has led to drugs called ‘antibody–drug conjugates’. In 2019 the FDA approved the antibody–drug conjugate enfortumab vedotin for breakthrough therapy designation on the basis of positive outcomes in a phase II trial[210,211]. This antibody–drug conjugate is administered intravenously and is approved for patients with locally advanced or metastatic bladder cancer who previously received an ICI and platinum-containing chemotherapy.

Immune checkpoint inhibitor regimens.—MIBC has one of the highest mutational profiles when compared with other cancers[212–214], and cancers with high mutational burden

such as melanoma[215] and non-small-cell lung cancer[216–218] respond very favourably to ICIs. Atezolizumab, a monoclonal antibody that binds PDL1, as opposed to the cognate receptor PD1 expressed on T cells, was approved for treating patients with MIBC. Recent findings from the IMvigor130 study[219] revealed the continued success of this agent for advanced bladder cancer. However, the FDA no longer considered the benefit–risk profile favourable for all cisplatin-ineligible patients. Therefore, in June 2018, the indication for both atezolizumab and pembrolizumab was modified to include only patients with MIBC who are not eligible for cisplatin-containing chemotherapy and who have high expression of PDL1 or are not eligible for any platinum-containing chemotherapy regardless of the level of PDL1 expression[220]. Most recently, the anti-PDL1 agent avelumab was approved for breakthrough therapy designation for first-line maintenance therapy for patients with MIBC who successfully responded to chemotherapy. Avelumab is also approved by the FDA for patients whose MIBC has advanced after chemotherapy. For MIBC there is currently no formal maintenance therapy, but this is where ICIs are likely to shine[3,221]. Avelumab has already shown efficacy against advanced and metastatic bladder cancer in ongoing phase II (REF.[222]) and phase III (REF.[223]) clinical trials, and was very recently approved as maintenance therapy for locally advanced or metastatic urothelial bladder cancer[224].

While the aforementioned approaches are showing great promise relative to past therapies, it must be recognized that response rates can still be increased. Two great challenges in this area include the development of robust companion biomarkers that can predict which patients will respond to specific ICI therapies, and assign the other patients to novel therapies being evaluated in clinical trials, and improvement on ICIs by developing novel combination therapies with targeted therapies, such as the FGFR inhibitors. Although a daunting challenge given the number of possible combinations with ICI, novel functional genomics approaches have the promise for high-throughput sampling of the combinatorial landscape to discover clinically tractable combinations. One such approach demonstrating the proof of principle of this concept has identified combining anti-PD1 treatment with the tyrosine kinase inhibitor dasatinib, as the therapies displayed synergy across several tumour types, including bladder cancer[225]. Further development of molecular marker analysis of patient response will allow future combination trials to select patients optimally to test these regimens rapidly and efficiently.

Conclusions and future directions

For decades there were few advancements in bladder cancer therapeutics. The last major advance was BCG administration, beginning in 1977. However, the past 7 years have seen unprecedented advances thanks mainly to patient tumour molecular profiling and check-point blockade immunotherapy. The former has led to a significant clinical utility in urine screening to assess bladder cancer recurrence, with several commercial clinical tests available. In addition to urine tests, molecular profiling can be used on blood samples, which would be useful for detecting recurrence. Checkpoint blockade immunotherapy has been a significant clinical advance in nearly all cancers. Most significantly, ICIs continue to displace previous treatment regimens as first-line and second-line therapies in bladder cancer, a sign of their significant potential and the more thorough understanding of their clinical applications. The challenges lying ahead include the development of companion

biomarkers to assign patients to the therapies they are most responsive to, and more effective combination therapy with checkpoint blockade immunotherapies, small molecules and other biologically based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 | Clinical application of molecular subtypes in bladder cancer

Molecular classification of tumours to identify the many different subtypes of bladder cancer (Supplementary Table 1) is defining tumours beyond just the classical basal or luminal type. With use of genetic signatures to better define bladder cancer tumours, reminiscent of breast cancer subtypes, luminal tumours were found to respond to treatment better than basal tumours[8]. The high mutational profile of basal tumours is the likely reason behind this fact, with a high degree of mutations in progrowth genes such as the ErbB family, allowing treatment with drugs such as afatinib[242]. No progrowth gene in bladder cancer has more activating mutations or amplified expression than the members of the fibroblast growth factor receptor (FGFR) gene family, most notably *FGFR3*. Molecular stratification by FGFR genetic status is key for treating patients with FGFR inhibitors such as erdafitinib or pemigatinib (for example, FIGHT-201 (REF.[243])). Subtyping tumours by inactivating mutations in DNA repair genes such as *ATM*, *RB1* and *FANCC* is also clinically applicable as these tumours exhibit a positive response to platinum-based neoadjuvant chemotherapy, with a much higher resolution coming following the completion of the current RETAIN clinical trial[244]. Identifying changes to epigenetic modifiers is another important molecular characterization for the clinic. DNA methyltransferase inhibitors such as decitabine[245] and guadecitabine[246] are being investigated for their ability to enhance the efficacy of cisplatin and atezolizumab, respectively. Beyond mutational assessment is gene expression assessment, where specific molecular subtypes exhibit differential responses to neoadjuvant chemotherapy and variable overall survival[11,34]. For patients with tumours of the basal subtype, luminal tumours with high expression of PDL1 or with tumours with high levels of tumour-infiltrating lymphocytes, there is the clinical option of being treated with one of the six immune checkpoint inhibitors approved for treating bladder cancer. Improved molecular subtyping and clinical utility of that information is currently being used in trials such as the BISCAY trial[247], which is allocating patients to one of seven modules, dependent on their genomic alterations. While clearly molecular subtyping has made its way into clinical decision-making, dramatic increases in molecular profiling of bladder cancer tumours need to be undertaken to the fullest extent possible, as even immature information can be assessed retrospectively to provide a significant benefit to future clinical applications.

Box 2 | The urinary microbiota

The human microbiota is the populations of bacteria, bacteriophages, fungi, protozoans and viruses that exist within and on the human body[248]. Only in the past decade have we begun to grasp the extent to which the microbiota facilitates carcinogenesis by modulating cell proliferation and/or death, immune function and metabolism of chemokines and pharmaceuticals[249]. Advances in high-throughput sequencing and culturing techniques have challenged previous dogma that urine is sterile and have established there also exists a urinary microbiota[250]. While few studies have explored the impact of the urinary microbiota on bladder cancer pathogenesis, it is theorized to be relevant to genitourinary cancers. Chronic genitourinary tract infections have been identified as a risk factor for bladder cancer due to their promotion of chronic

inflammation[251]. The clearest example of this occurrence is the link between a history of schistosomiasis infections and bladder squamous carcinoma. *Schistosoma haematobium* parasitic blood flukes lay eggs in the urinary bladder, inducing chronic cystitis and fibrosis, which contribute to tumorigenesis[252]. Analysis of the urinary microbiota in those infected with *S. haematobium* or those who presented with abnormal bladder pathology concurrent with *S. haematobium* infection revealed taxa different from those in healthy persons. Of note, the phyla Proteobacteria and Firmicutes primarily compose the urinary microbiota, and five genera were differentially abundant in infected participants with and without induced bladder pathology: *Facklamia*, *Veillonella*, *Fusobacterium*, *Bacteroides* and *Aerococcus*[253]. Several studies have verified an altered microbiota in patients with bladder cancer, with increased abundance of genera such as *Streptococcus*, *Pseudomonas*, *Anaerococcus*, *Fusobacterium*, *Acinetobacter* and *Sphingobacterium*[254–256]. *Herbaspirillum*, *Porphyrobacter* and *Bacteroides* were enriched in patients with bladder cancer with high risk of recurrence and progression[257]. Unfortunately, studies to date have not been able to fully determine if these bacterial taxa are directly contributing to bladder disease or are opportunistically colonizing altered tissue. The gut microbiota field is far more developed, but few studies have included bladder cancer in their purview. Notably, antibiotic-induced imbalances in microbial communities (dysbiosis) was shown to affect the therapeutic efficacy of immune checkpoint inhibitors, with patients with bladder cancer having significantly shorter progression-free and overall survival[258]. On a positive therapeutic front, oral administration of a common probiotic, *Lactobacillus casei*, in conjunction with intravesical instillation of epirubicin following transurethral resection, increased 3-year recurrence-free survival from 60% to 75% in patients with non-muscle-invasive bladder cancer[259].

Cystoscopy

A process whereby an imaging device is inserted into a patient's urethra for the purpose of imaging the urethra and bladder lining to look for abnormalities.

Uroplakins

Protein complexes normally expressed on the surface of luminal cells of the bladder and that are an indicator of terminal differentiation. Loss of uroplakin III expression is therefore indicative of disease progression and is correlated with poor prognosis.

Regulons

Groups of genes regulated as a unit and controlled by the same regulatory gene that expresses a protein acting as a repressor or activator.

Gene promoter

sequence of DNA to which RNA polymerase and transcription factors bind to initiate RNA synthesis of a gene.

Telomere

Cap made of a repetitive nucleotide sequence at the ends of a chromosome to protect it from deterioration.

Plasmacytoid variant

Infiltrating urothelial carcinoma that is characterized by tumour cells that have a striking morphologic resemblance to and immunohistochemical overlap with plasma cells, and that harbours a *CDH1* mutation.

Nested variant

A rare neoplasm that is histologically characterized by large numbers of small, closely packed, haphazardly arranged nests of urothelial cells infiltrating the lamina propria and the muscularis propria.

Papillary urothelial carcinomas

Tumours with thin, finger-like growths that start in the bladder lining and extend into the centre of the bladder.

Glandular lesions

An intriguing group of clinically and morphologically diverse neoplasms, encompassing benign processes to malignant growths.

Inverted papillomas

Rare yet benign neoplasms that is are moderate significance due to their similarity to inverted urothelial carcinoma, which has a much more aggressive prognosis.

Complex of proteins associated with Set1

(COMPAss). Methyltransferase protein complex that controls gene transcription by specifically methylating histone H3 on the lysine at position 4 of the protein.

Immune checkpoint inhibitors

(ICIs). A class of therapies (antibodies) that reactivate dormant or exhausted cytotoxic T cells.

Intravesical instillations

Administration of therapeutics directly to the bladder through a soft catheter inserted through the urethra.

Bacillus Calmette–Guérin

(BCG). A live attenuated strain of *Mycobacterium bovis*. Used as a vaccine against tuberculosis (first given to patients in 1921). since 1977, it has been used as an immunotherapeutic agent in patients with bladder cancer.

Radical cystectomy

Complete removal of the bladder in cases of advanced muscle-invasive bladder cancer with the aim of removing bladder cancer entirely from the patient.

Androgen deprivation therapy

A clinical approach to inhibit signalling of the hormone androgen, either by chemical or surgical castration, with the goal of preventing androgen-dependent cancer cells from growing.

5α-Reductase

Enzyme that catalyses conversion of testosterone to dihydrotestosterone.

Hydroxyflutamide

An active metabolite of flutamide used as an androgen antagonist.

Urine cytology

A process whereby cells from a patient’s urine are collected and analysed under a microscope to look for cellular abnormalities such as those found in bladder cancer.

Metastatic relapse

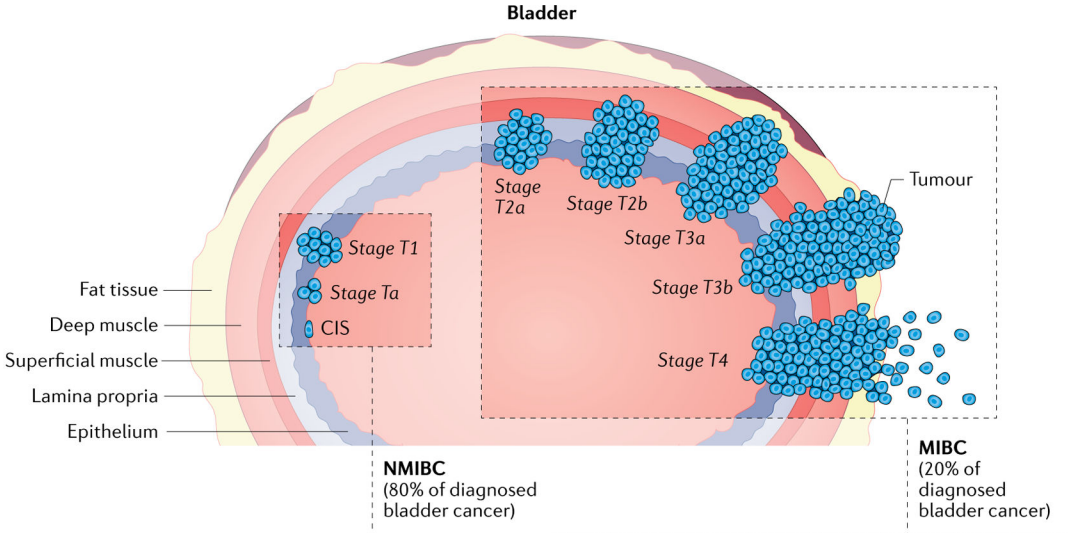
Relapse of cancer after treatment with the cancer spreading to distant organs.

Transurethral resection of the bladder

A surgical technique used to harvest tissue samples for pathology analysis and remove non-muscle-invasive bladder cancer tumours.

Photodynamic therapy

A therapy involving localized activation of photosensitive molecules that are selectively taken up by cancer cells, reducing toxicity to normal cells.



Subtype	Signatures	Mutations
Class 1 (20%, 97/476)	• PPARG$^+$ • UPK$^+$ • Early cell cycle genes	*FGFR3*
Class 2 (52%, 249/476)	• Luminal-like differentiation • PPARG$^+$ • UPK$^+$ • KRT14$^+$ • CIS positive • EMT transcription factors • Cancer stem cell activity • Late cell cycle genes • APOBEC$^+$ signature	• *TP53* • *ERCC2*
Class 3 (27%, 130/476)	• Basal-like undifferentiation • PPARG$^-$ • GATA3$^+$ • KRT5$^+$ • KRT14$^+$ • KRT15$^+$ • CD44$^+$ • RNA-editing signature	*FGFR3*

Subtype	Signatures	Mutations
LumP (24%)	• PPARG$^+$ • FGFR3$^+$ • CDKN2A$^-$	• *FGFR3* (40%) • *KDM6A* (38%)
LumNS (8%)	PPARG$^+$	*ELF3* (35%)
LumU (15%)	• PPARG$^+$ • E2F3$^+$ • ERBB2$^+$ • Genomically unstable • Cell cycle positive • APOBEC$^+$ • High TMB	• *TP53* (76%) • *ERCC2* (22%)
Stroma-rich (15%)	• Smooth muscle • Fibroblast • Myofiblast gene signatures	–
Ba/Sq (35%)	• Squamous differentiation markers • Fibroblasts and myofibroblast gene signature • EGFR$^+$	• *TP53* (61%) • *RB1* (25%)
NE-like (3%)	• Neuroendocrine differentiation marker • TP53$^-$ • RB1$^-$ • Cell cycle positive	• *TP53* (94%) • *RB1* (39%)

Fig. 1 |. Pathological and molecular features of human bladder cancer.

Urothelial carcinoma of the bladder is comprised of two major groups on the basis of clinical staging with different clinical outcomes and therapy options: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Staging of urothelial bladder carcinoma is shown according to the tumour, lymph node, metastasis (TNM[240]) system. The UROMOL study classified NMIBC into three classes: class 1, luminal-like signature; class 2, luminal-like, epithelial–mesenchymal transition (EMT) and cancer stem cell signatures; and class 3, basal-like signature. Six subgroups of MIBC are shown: luminal papillary (LumP), luminal non-specified (LumNS), luminal unstable (LumU), stroma-rich, basal/squamous (Ba/Sq) and neuroendocrine-like (NE-like). APOBEC, apolipoprotein B mRNA editing catalytic polypeptide-like family of proteins; FGFR3, fibroblast growth factor receptor 3; UPK, uroplakin; TMB tumour mutation burden.

Fig. 2 |. Major genomic alterations in human bladder cancer.

Whole-transcriptome mRNA profiling of patient samples identified frequently altered genes and pathways that drive bladder cancer (The Cancer Genome Atlas (TCGA)[241]). The data shown in this figure were retrieved from TCGA Pan Cancer Atlas in CBioPortal, and represent the frequency of altered genes in patient samples (n = 406) and the proportions of the different types of alterations per gene. Somatic alterations of genes are most common in pathways related to p53, the cell cycle, receptor tyrosine kinase (RTK)–RAS–PI3K and epigenetic modifications. Alterations in *TP53* and cell cycle genes are common in muscle-invasive bladder cancer (MIBC), including *CDKN2A*, *CDKN2B*, *RB1*, *STAG2*, *E2F3*, *ATM*, *CDKN1A*, *ERCC2*, *MDM2* and *FBXW7*. Common mutations for *TP53* are missense and truncating mutations (frameshift, insertion/deletion and splice site mutation), which induce loss of funtion. Genes limiting cell cycle activity such as *CDKN2A*, *CDKN2B*, *RB1* and *CDKN1A* are commonly inactivated by homologous deletion and truncating mutations. Progrowth signalling genes *E2F3* and *MDM2* commonly exhibit amplification. *STAG2* encodes a subunit of the cohesin complex, which regulates sister chromatid separation during cell division and is frequently silenced by truncating mutations in MIBC. *ATM* encodes a DNA damage sensor protein and exhibits loss of function when mutated (mostly missense and truncating mutations) in 14% of patients with MIBC. The protein product of *ERCC2* is involved in nucleotide excision repair, *ERCC2* is mutated in 11% of patients with MIBC and missense mutations drive MBIC cisplatin sensitivity. *FBXW7* encodes a

protein that functions in cell cycle exit and stem cell maintenance and is often found mutated in MIBC. Activating mutations in *FGFR3*, *PIK3CA*, *ERBB2* and *ERBB3* enhance tumour growth. TSC1 negatively regulates mechanistic target of rapamycin complex 1 signalling and the gene is frequently inactivated by truncating mutations. Bladder cancer often has truncating and missense mutations in chromatin remodelling genes such as *ARID1A*, *KDM6A*, *KMT2D*, *KMT2C*, *EP300*, *CREBBP* and *ASXL2*.

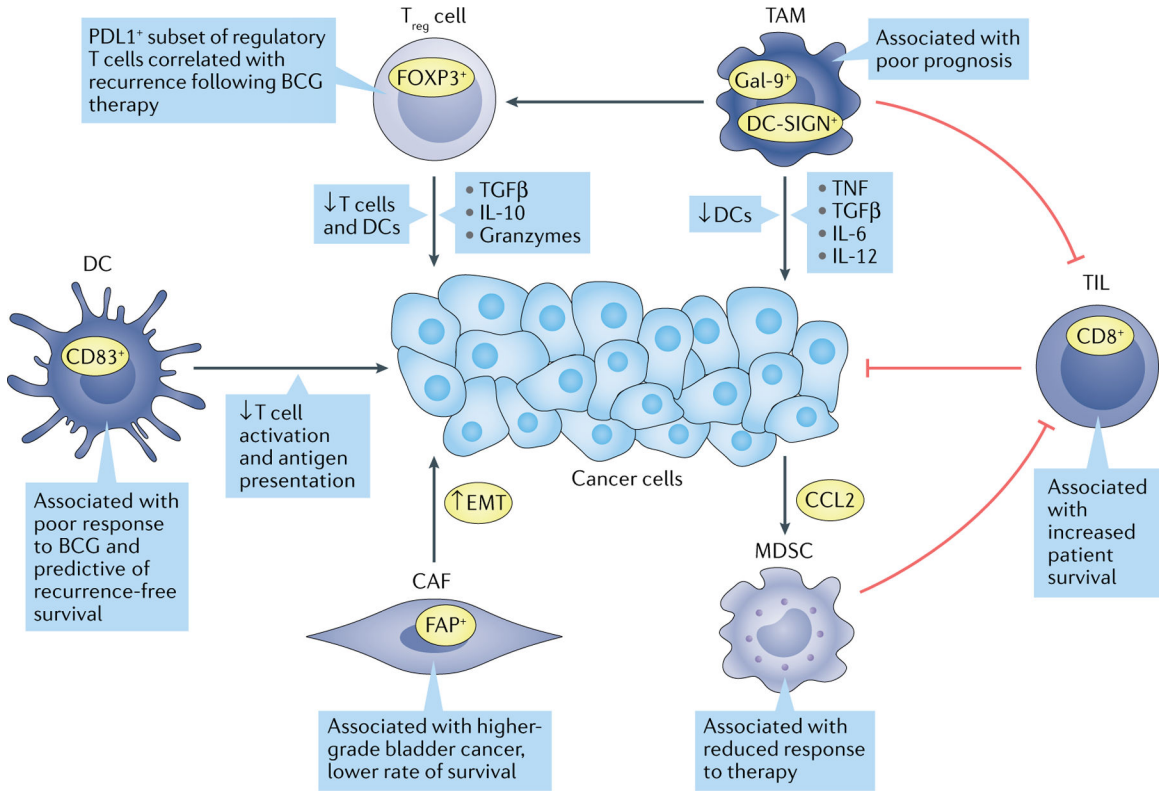


Fig. 3 |. Non-tumour cell types implicated in bladder cancer progression.

Signalling between cancer, stromal and immune cells modulates tumour growth and aggressiveness. Many of these cell types have been correlated with therapeutic resistance and lower survival outcome for patients. Green arrows indicate protumorigenic associations, while red bars indicate antitumour activity. CAF, cancer-associated fibroblast; CCL2, C-C motif chemokine ligand 2; DC, dendritic cell; DC-SIGN, dendritic cell-specific C-type lectin; EMT, epithelial–mesenchymal transition; FAP, fibroblast activation protein; FOXP3, forkhead box protein P3; Gal-9, galectin 9; MDSC, myeloid-derived suppressor cell; TAM, tumour-associated macrophage; TIL, tumour-infiltrating lymphocyte; TGFβ, transforming growth factor-β; TNF, tumour necrosis factor; T_{reg} cell, regulatory T cell.

Table 1 |

Preclinical models used in discovery and translation

Model	Description	Relevance	Limitations	Refs
Urinary tract induced pluripotent stem cells	Human induced pluripotent stem cells are generated from urinary tract tissue	Used to characterize mechanisms regulating bladder differentiation and model disease	Viruses used to induce embryonic genes may cause cancer. Methods have not been extensively validated	[226]
Patient-derived organoids	Maintenance of bladder cancer cells in 3D cultures	Cell–cell and cell–matrix interactions are more similar to tumours, allowing more accurate high-throughput drug screening	Models have not been extensively validated. Heterogeneity of cells can hinder reproducibility	[227,228]
Patient-derived xenograft	Patient tumour samples implanted into immunodeficient mice	Models specific patient samples and subtypes. NCI has developed a national repository. Humanized mice improve therapeutic opportunity	Cannot query immune mechanisms. Limited translation to patients	NCI Patient-Derived Models Repository (NCI at Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD, USA)
Renal engraftment	Combination of bladder cancer cells with embryonic mesenchyme under the renal capsule of immunocompromised mice	Incorporates stromal–epithelial interactions in functional studies	Microenvironment does not recapitulate bladder tissue	[229]
Orthotopic engraftment	Human bladder cancer cells implanted into the bladder wall of immunodeficient mice	Microenvironment more closely models bladder disease	Tumours do not develop de novo. Cannot query immune mechanisms	[230]
Syngeneic engraftment	Murine bladder cancer cells implanted into an immunocompetent syngeneic host	Ease of analysis for immunotherapeutic targets	Microenvironment does not recapitulate bladder tissue	[225]
Carcinogen induced	Administration of carcinogens through drinking water or intravesical instillation. Common agents include *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and *N*-methyl-*N*-nitrosourea	Tumours develop de novo from a carcinogen known to be associated with human bladder cancer	Cannot probe specific molecular subtypes due to heterogeneity. Substantial investment of time for tumour development	[231]
Genetically engineered mouse	Expression of oncogenes and/or knockout of tumour suppressors in the urothelium/germ line	Models common mutations observed in human bladder disease	Substantial investment of time for model development. Limited targeting of genes rarely recapitulates complexity of disease	[232]

A comprehensive review of murine and human bladder cancer cell lines has recently been published[233] and is not reiterated here. NCI, US National Cancer Institute.

Table 2 |

Currently available non-invasive molecular diagnostics

Studies	Materials	Targets	Number of patients per study	Refs
UroMuTERT	Urinary cellular DNA and cfDNA	*TERT* promoter	93 patients with bladder cancer and 94 controls	234
1 gene	cfDNA	*TERT* promoter	53 patients with bladder cancer and 36 controls; 104 patients with bladder cancer	146,235
2 gene	cfDNA	*TERT* promoter and *FGFR3*	153 patients with bladder cancer and controls	236
UroSEEK	Urinary cellular DNA	*TERT* promoter region (TERTSeqS) + 10 genes (*FGFR3, PIK3CA, TP53, HRAS, KRAS, ERBB2, CDKN2A, MET, MLL* and *VHL*) (UroSeqS)	570 patients with bladder cancer; 527 patients with bladder cancer	145
Somatic mutation, 23 genes	Urinary cellular DNA and cfDNA	*TERT* promoter, *FGFR3, PIK3CA, TP53, ERCC2, RHOB, ERBB2, HRAS, RXRA, ELF3, CDKN1A, KRAS, KDM6A, AKT1, FBXW7, ERBB3, SF3B1, CTNNB1, BRAF, C3orf70, CREBBP, CDKN2A* and *NRAS*	956 patients with bladder cancer	237
Stanford University uCAPP-Seq	Urinary cellular DNA and cfDNA	High-throughput targeted sequencing approach	118 patients with bladder cancer and 67 controls	238
Hong Kong methylomic and copy number analysis	cfDNA	Shallow-depth paired-end genome-wide bisulfite sequencing	46 patients with bladder cancer and 39 controls	40
5 gene + 7 gene panel	Urinary cellular DNA and cf DNA	5 genes for urine supernatant cfDNA (*TERT, FGFR3, TP53, PIK3CA*, and *KRAS*); 7 genes for urine sediment cellular DNA (*TERT, FGFR3, TP53, HRAS, PIK3CA, KRAS* and *ERBB2*)	16 patients with bladder cancer	239

cfDNA, cell-free DNA.

Table 3 |

Clinical trials discussed in this Review

Interventions	Administration type	Phase	Start date	Completion date	Status	Recruiting?	Number of patients	Ref.
Non-muscle-invasive disease [a]								
TAR-200 drug delivery system (gemcitabine); nivolumab	Intravesical; systemic	I	Jan. 2019	Sep. 2020	Completed	No	25	162
TAR-200 drug delivery system (gemcitabine)	Intravesical	I	Jan. 2018	Mar. 2020	Completed	No	35	163
TC-3 gel (mitomycin C)	Intravesical	II	Dec. 2014	Jun. 2017	Completed	No	14	164
UGN-102 (gemcitabine)	Intravesical	II	Oct. 2018	Nov. 2020	Active	No	63	165
TLD1433 infusion and photodynamic therapy	Intravesical	I	Dec. 2016	Aug. 2018	Completed	No	6	170
TLD1433 infusion and photodynamic therapy	Intravesical	II	Aug. 2019	May 2022	Active	Yes	125	172
Pembrolizumab	Systemic	II	Feb. 2016	Jul. 2023	Active	Yes	260	182
Attenuated measles virus (MV-NIS)	Intravesical	I	Jul. 2018	Apr. 2021	Active	Yes	16	183
Toca 511	Intravesical	I	Dec. 2019	NA	Terminated	No	0	184
Nadofaragene firadenovec (adenovirus vector; interferon-α_{2b} gene)	Intravesical	III	Sep. 2016	Aug. 2022	Active	No	157	185
Ty21a (typhoid vaccine)	Intravesical	I	Feb. 2018	Mar. 2021	Active	Yes	25	186
Recombinant EphB4–HSA fusion protein	Intravesical	I	May 2020	May 2023	Active	Not yet	36	187
ALT-803 (IL-15 receptor-α fusion); BCG	Intravesical	II	Jun. 2017	Jan. 2023	Active	Yes	183	188
BC-819 (plasmid expressing diphtheria toxin in malignant cells)	Intravesical	II	Dec. 2018	Aug. 2020	Terminated	No	140	189
Muscle-invasive disease [b]								
COXEN; 75 approved agents	Various	II	Mar. 2017	Oct. 2019	Completed	No	8	200
COXEN; chemotherapy	Various	II	Jul. 2014	Oct. 2022	Active	No	237	201
Enfortumab vedotin (antibody–drug conjugate targeted at nectin 4)	Systemic	II	Oct. 2017	May 2025	Active	No	219	211
Atezolizumab	Systemic	III	Jun. 2016	Jun. 2021	Active	No	1,200	219
Avelumab + gemcitabine/carboplatin	Systemic	II	May 2018	Aug. 2020	Active	No	85	222
Avelumab	Systemic	III	Apr. 2016	Jun. 2023	Active	No	700	223

Clinical trials discussed in the text are listed here in order of discussion. BCG, bacillus Calmette–Guérin; COXEN, coexpression extrapolation; EphB4, ephrin type B receptor 4; HSA, human serum albumin; NA, not applicable.

[a] For non-muscle-invasive bladder cancer, the most promising interventions are administered via intravesical instillation, except for immune blockade therapy, which is given systemically intravascularly.

[b] For muscle-invasive bladder cancer, the administrations are all given systemically as the disease has spread beyond the bladder lumen.