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Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity

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Preface

Urothelial carcinoma of the bladder comprises two long-recognised disease entities with distinct molecular features and clinical outcome. Low-grade, non-muscle-invasive tumours recur frequently but rarely progress to muscle invasion, whereas muscle invasive tumours are usually diagnosed *de novo* and frequently metastasize. Recent genome-wide expression and sequencing studies identify genes and pathways that are key drivers of urothelial cancer and reveal a more complex picture with multiple molecular subclasses that cut across conventional grade and stage groupings. This improved understanding of molecular features, disease pathogenesis and heterogeneity provides new opportunities for prognostic application, disease monitoring and personalised therapy.

Bladder cancer is the most common cancer of the urinary tract with approximately 380,000 new cases and 150,000 deaths per year worldwide[1]. It ranks fifth among cancers in men in Western countries. Epidemiological studies identify a range of environmental risk factors, many of which reflect exposure to excreted carcinogenic molecules (BOX 1). Recent genome-wide association studies have also identified germline variants that contribute to risk[2].

In Europe and North America, more than 90% of bladder cancers are urothelial carcinoma. These tumours are staged using the Tumour Nodes Metastasis (TNM) system[3], which describes the extent of invasion (Tis-T4), and graded according to their cellular characteristics. Two classification systems are in current use[4, 5]. At diagnosis the majority of bladder cancers (~ 60%) are non-muscle-invasive (NMIBC) (stage Ta) papillary tumours of low grade (Figure 1). Stage T1 tumours, which have penetrated the epithelial basement membrane but have not invaded muscle, are mostly of high grade as are muscle-invasive (MIBC) tumours (~20% at diagnosis).

NMIBCs frequently recur (50-70%) but infrequently progress to invasion (10-15%)[6] and five-year survival is ~90%. These patients are monitored by cystoscopy and may have multiple resections over many years. Improved monitoring is needed, ideally via urine analysis, which could reduce the morbidity and costs associated with cystoscopy. Although risk tables provide a prognostic tool[7], no molecular biomarkers accurately predict disease progression. For these patients, localised therapies to remove residual neoplastic and preneoplastic cells post-resection may have major impacts on both quality of life and in health economic terms. MIBCs ($\geq$stage T2) have less favourable prognosis with five-year survival $\leq$50% and common progression to metastasis (BOX1). Treatment has not advanced for several decades and new approaches to systemic therapy are needed[8].

Improved treatment requires detailed understanding of urothelial carcinoma pathogenesis and molecular biology. A model has evolved, taking into account both histopathological and molecular features. This so-called 'two-pathway' model proposes that papillary NMIBC develops via epithelial hyperplasia and recruitment of a branching vasculature. MIBCs are proposed to develop via flat dysplasia and carcinoma *in situ* (CIS). The molecular characteristics of MIBC and NMIBC are highly distinct (Tables 1 and 2). Whilst

many features of bladder cancer fit well within this model, there is considerable heterogeneity in clinical behaviour. This Review discusses the molecular features of bladder tumours and recent findings that begin to unravel this heterogeneity and pave the way for a step change in personalised patient care.

The molecular landscape

Genomic instability, chromosomal alterations and allelic loss. NMIBCs commonly have near-diploid karyotype and few genomic rearrangements. By contrast, MIBCs are commonly aneuploid with many alterations including chromothripsis[9]. Non-homologous end joining is implicated as a mechanism for error-prone double strand break repair in MIBC[9, 10]. Mutation of minichromosome maintenance complex component 4 (*MCM4*), a component of the replication-licensing complex in some MIBCs has led to the suggestion that failure to rescue stalled replication forks may underlie complex translocation events involving multiple chromosomes[9]. Inactivating mutations are reported in DNA repair and DNA damage checkpoint genes including excision repair cross-complementation group 2 (*ERCC2*), ataxia-telangiectasia mutated (*ATM*) and Fanconi anaemia complementation group A (*FANCA*) in MIBC. Frequent mutation of stromal antigen 2 (*STAG2*), a component of the cohesin complex, has been identified, with higher frequency in NMIBC[11-13]. Cohesin plays a role in chromatid segregation and in other tumour types *STAG2* inactivation is associated with aneuploidy[14]. In bladder cancer there is no clear relationship to aneuploidy[11, 13] and as cohesin also plays a role in genomic organisation via interaction with CCCTC-binding factor (CTCF)[15] and in DNA double strand break repair by homologous recombination[16], these functions may be more important. Several other genes involved in sister chromatid cohesion and segregation (*STAG1,* nipped-B homologue (*NIPBL*), structural maintenance of chromosomes 1A (*SMC1A*), *SMC1B, SMC3,* extra spindle pole bodies homologue 1 (*ESPL1*)), show mutation in MIBC[11, 17].

Such defects lead to a variety of chromosomal alterations. Chromosome 9 deletion is common in both NMIBC and MIBC (>50%). Candidate tumour suppressor genes affected by chromosome 9 deletion are cyclin-dependent kinase inhibitor 2A (*CDKN2A*; which encodes p16 and $p14^{ARF}$) and *CDKN2B* (which encodes p15) at 9p21[18, 19], patched 1 (*PTCH1*; at 9q22)[20, 21], deleted in bladder cancer 1 (*DBC1*; also known as *BRINP1*; at 9q32-33)[22, 23] and tuberous sclerosis 1 (*TSC1*; at 9q34)[24, 25]. Loss of heterozygosity (LOH) of 9p, homozygous deletion of *CDKN2A* and loss of expression of p16 in NMIBC predicts reduced recurrence-free interval[26-28]. As mouse knockouts and *in vitro* experiments[29, 30] suggest that p16 and/or $p14^{ARF}$ are haploinsufficient tumour suppressors, it is plausible that the loss of one allele in ~45% of bladder cancers has functional consequences. Importantly, in the small group of MIBC with fibroblast growth factor receptor 3 (*FGFR3*) mutation (discussed below), a high frequency of *CDKN2A* homozygous deletion has been reported[31]. This may identify a progression pathway for *FGFR3*-mutant NMIBC to muscle invasion. Loss of p16 expression is inversely correlated with RB1 expression[32] and conversely, high-level expression results from negative feedback in tumours with RB1 loss[33]. Both changes are adverse prognostic biomarkers, found in >50% of MIBC[34].

The best-validated 9q tumour suppressor gene is *TSC1*. The TSC1-TSC2 complex negatively regulates the mTOR branch of the PI3K pathway (Figure 2). A recent study has also identified mutations in *NOTCH1* in 18% of tumours[35]. However, no 9q genes show biallelic mutational inactivation at frequencies that are compatible with the high frequency of LOH. Indeed, no chromosome 9 genes show significant mutation frequency in exome sequencing studies[11, 17, 36, 37]. This may indicate haploinsufficiency for one or more genes or implicate epigenetic rather than genetic mechanisms of inactivation. After more than 2 decades, the quest to identify drivers of 9q loss in bladder cancer continues.

Other copy number changes and allelic loss in bladder cancer have been identified by comparative genomic hybridization (CGH) and LOH analyses, though many target genes remain unknown[38-40]. Regions of deletion associated with aggressive disease are 8p, 2q and 5q[41-43]. In addition to amplicons containing known oncogenes (Table 1), amplicons on 1q21-q24, 3p25, 6p22, 8p12-p11, 11p15, 11q14, 12q24, 20q12-q13 have been identified by CGH[43-45] and in addition to homozygous deletion of *CDKN2A,* homozygous deletion of regions on 9p21.3, 2q36, 11p11, 18p11 and 19q12 have been reported. Copy number data from low-pass whole genome sequence and/or single nucleotide polymorphism (SNP)-array analysis of MIBC has also identified amplicons on 19q, 1q22-q23, 8p11 and 20q11, and deletions on 2q21, 2q34, 4q22, 5q12*,* 6p25 and 16p13[17, 37]. Although MIBCs show many chromosome rearrangements, the only recurrent gene-gene fusion identified is *FGFR3*-transforming, acidic coiled-coil containing protein 3 (*TACC3*)[37, 46].

Mutation frequency and signature. Exome sequence from 294 bladder tumours, largely MIBC or stage T1, has been reported[11, 17, 36, 37, 47]. Somatic mutation frequency in MIBC is reported as ~300 exonic mutations per sample, with mean and median rates of 7.7 and 5.5 per megabase[37, 48], a frequency exceeded only by lung cancer and melanoma and dominated by C:G>T:A transitions. A tobacco smoke (polycyclic hydrocarbon)-exposure signature (C:G>A:T) is not apparent. Interestingly, many C:G>T:A mutations are found in the context TpC[48], a pattern that is characteristic of mutations caused by the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family of cytidine deaminases, enzymes that normally restrict the propagation of retroviruses and retrotransposons, and which deaminate cytosine, leaving this tell-tale signature[49]. Indeed, *APOBEC3B* expression is significantly upregulated in human bladder cancer[37, 50].

FGFR alterations. Up to 80% of stage Ta tumours have activating point mutations in *FGFR3* (Figure 3A) and this is associated with favourable outcome[51-57]. In stage T1 tumours and MIBC, *FGFR3* mutation is less common (10-20% in $\geq$T2)[58-60]. In cultured normal human urothelial cells (NHUCs), mutant FGFR3 activates the RAS-MAPK pathway and phospholipase Cγ (PLCγ), leading to increased survival and proliferation to high cell density[61]. This *in vitro* phenotype suggests that *FGFR3* mutation could contribute to early clonal expansion within the urothelium *in vivo.*

FGFR3 is also implicated in the risk of bladder cancer development. A SNP in an intron of *TACC3,* 70 kb from *FGFR3* is associated with bladder cancer risk[62], and with higher risk of recurrence in stage Ta disease,

particularly for *FGFR3*-mutant NMIBC. The mechanism (or mechanisms) underlying these links is not clear but one possibility is that altered chromatin structure associated with increased expression could increase the probability of mutation and/or increase the expression and impact of mutations that occur.

In normal urothelium, FGFR3 is expressed as the IIIb isoform, which mainly binds FGF1. A splice variant (Δ8-10) encodes a secreted form that lacks the transmembrane domain, which may act as a negative regulator by sequestering FGFs or binding full-length receptors[63]. In bladder cancer cell lines, reduced expression of full-length FGFR3-IIIb and FGFR3-Δ8-10 isoforms and a switch to the FGFR3-IIIc isoform, which binds many FGF ligands[64], may facilitate autocrine or paracrine signalling[63]. Furthermore, many bladder cancers, including those without *FGFR3* point mutation, show increased expression of FGFR3[60]. MicroRNAs (miRNAs) miR-99a and miR-100, which negatively regulate the expression of FGFR3, are also commonly downregulated in bladder cancer, particularly in NMIBC[65].

Some cell lines and tumours (~5%) contain chromosomal translocations that generate FGFR3 fusion proteins. These fusions comprise amino acids 1-760 of FGFR3 (IIIb isoform, including the kinase domain), fused in-frame to *TACC3*[17, 37, 46] or BAI1-associated protein 2-like 1 (*BAIAP2L1*)[46]. These are highly activated and transforming oncogenes. An additional mechanism for the activation of FGFR3 in bladder cancer could include upregulated expression of FGF ligands by tumour cells or stroma, although this has not been adequately assessed (Figure 3B).

FGFR1 expression is upregulated in both NMIBC and MIBC[66], although no mutations have been reported. An increased ratio of the FGFR1β:FGFR1α splice variants is found in tumours of higher grade and stage[66]. The β isoform, lacking the first extracellular immunoglobulin-like domain, shows increased sensitivity to FGF1[67] (Figure 3B). FGF2 stimulation of FGFR1β in NHUCs activates the MAPK pathway and PLCγ, leading to increased proliferation and reduced apoptosis[66]. Similar stimulation in bladder cancer-derived cell lines can induce an epithelial-mesenchymal transition (EMT), a major feature of which is PLCγ-mediated upregulation of cyclooxygenase 2 (COX2; also known as PTGS2)[68]. In accord with this, bladder cancer cell lines with the highest FGFR1 expression show a mesenchymal (EMT) phenotype (indicated by low E-cadherin expression) and upregulated FGF2 expression, and those with epithelial phenotype show higher FGFR3 and E-cadherin expression[69].

PI3K pathway alterations. The PI3K pathway is activated by several mechanisms (Figure 2). Some events are not mutually exclusive, implying non-redundant or non-canonical functions in bladder cancer[24]. Upstream activators include ERBB receptors. ERBB3 interacts with p110α, the catalytic subunit of PI3K, conveying signals from ERBB2-ERBB3 heterodimers. Epidermal growth factor receptor (EGFR; also known as ERBB1) induces PI3K activation via RAS activation. Overexpression of EGFR, ERBB2 and/or ERBB3 in subsets of bladder cancer, is associated with grade, stage and outcome[70] and ERBB2 and ERBB3 are mutated in some MIBCs[36, 37]. ERBB2 amplification or over-expression is more common in metastases than in the corresponding primary tumour, implying a role in the metastatic process[71, 74]. The receptor tyrosine kinases

MET and RON (also known as MST1R), activation of which also activates the PI3K pathway, are upregulated in aggressive bladder cancer[75-77]. It is not yet clear whether FGFR3 is a major PI3K pathway activator. Although mutant FGFR3 does not activate the pathway in NHUCs[61], higher levels of phosphorylated AKT (which indicates it is activated) have been reported in FGFR3 mutant tumours than in FGFR3-wild-type tumours[78].

Activating mutations of PIK3CA (which encodes p110α), more commonly in the helical domain (E545K and E542K) than in the kinase domain (H1047R), are found in ~25% of NMIBCs and less frequently in MIBC[24, 25, 78-80]. E542K and E545K mutants require interaction with RAS-GTP but not binding to the PI3K regulatory subunit p85, whereas p110α–H1047R depends on p85 binding but not RAS-GTP binding[81]. Possibly helical domain mutant proteins co-operate with events that activate RAS in bladder cancer. The expression of mutant PIK3CA confers a proliferative advantage at confluence and stimulates intraepithelial movement in NHUCs, with higher activity of helical domain mutants[82].

The lipid and protein phosphatase PTEN negatively regulates PI3K. PTEN commonly shows LOH in MIBC[83-85], but biallelic inactivation is uncommon. Overall, 46% of bladder cancer cell lines (largely from MIBC), had PTEN alterations[24]. Downregulated expression of PTEN in MIBC is associated with TP53 (which encodes human p53) alteration and poor outcome[24, 86]. Compatible with this, urothelial Trp53 (which encodes mouse p53) and Pten dual deletion but not deletion of either gene alone, leads to the development of metastatic bladder cancer in mice[86].

The protein phosphatase activity of PTEN influences cell motility. A mutant form, PTEN-G129E, that is deficient in lipid but not protein phosphatase activity, inhibits the invasive phenotype of the PTEN-mutant T24 bladder cancer cell line[87], implying a more important role for PTEN protein phosphatase activity in invasive bladder cancer. This is compatible with the finding that only PIK3CA mutation, which is predicted to phenocopy loss of PTEN lipid phosphatase activity, occurs in NMIBC.

Other pathway alterations include *TSC1* mutation (discussed above) and several mutations reported at low frequency (Tables 1 and 2).

Activation of the MAPK pathway. The role of MAPK signalling (Figure 2) and its relationship to key mutations in bladder tumours are not clear. RAS gene (*HRAS* or *KRAS*) and *FGFR3* mutations are mutually exclusive in bladder cancer. Mutation of one or the other in >82% of NMIBC[88] may suggest a similar function. However it is likely that there are also non-redundant functions as RAS gene mutation is relatively infrequent compared to *FGFR3* mutation and unlike *FGFR3* mutation, RAS gene mutation occurs at a similar frequency in NMIBC and MIBC. Although mutant FGFR3 activates MAPK pathway but not PI3K pathway signalling in NHUCs[61], immunohistochemistry for phosphorylated ERK (an indicator of MAPK pathway activation) does not show a strong relationship with *FGFR3* mutation or expression in tumour tissues[78, 89] and at least some bladder cancer cell lines are less sensitive to MEK inhibitors than NHUCs[90]. Whilst the common finding of *FGFR3* mutation with *PIK3CA* mutation in NMIBC[78, 80] suggests co-operative

activation of MAPK and PI3K pathways, the exact role of *FGFR3* in activation of the MAPK pathway requires further clarification. The recent finding of inactivating mutations in NOTCH pathway genes implicates this pathway in ≥40% of bladder cancers[35], suggesting that in this cell context the pathway has a tumour suppressor role. Higher levels of phosphorylated ERK1 and ERK2 were found in tumours with pathway alterations than in *FGFR3* or RAS-mutant tumours. This was mediated via reduced expression of several dual specificity protein phosphatase (DUSP) genes that target phosphorylated ERK, which are regulated by the intracellular domain of NOTCH1 (N1ic). Exogenous expression of N1ic or the ligand Jagged 1 (JAG1) in bladder cancer cells reduced phosphorylated ERK levels and inhibited proliferation. Overall, current data suggest that a majority of bladder cancers may be highly ERK1- and ERK2-dependent.

Hedgehog and WNT signalling. Several components of the canonical WNT signalling pathway are altered in bladder cancer. Low frequencies of mutation in adenomatous polyposis coli (*APC*) and β-catenin (encoded by *CTNNB1*) have been reported and reduced β-catenin protein expression or increased nuclear localisation is frequent in MIBC[25, 91-94]. Epigenetic silencing of the WNT antagonists, secreted frizzled receptor proteins (SFRPs)[95], and WNT inhibitory factor 1 (WIF1)[96] have also been reported.

The importance of WNT signalling is confirmed in mouse models. Expression of activated β-catenin in suprabasal urothelial cells in conjunction with *Pten* deletion led to bladder cancer development. Correlation between nuclear β-catenin, reduced PTEN and increased levels of phosphorylated AKT in human bladder cancer, indicate that similar co-operation is likely[97]. Another study that expressed active β-catenin in urothelial basal cells reported development of papillary tumours, more commonly in male mice. Synergy between β-catenin and androgen receptor signalling was demonstrated, implicating these factors in the observed sexual dimorphism of bladder cancer[98]. In mice, co-operation between β-catenin and mutant *Hras* is also reported, with MAPK rather than PI3K pathway signalling activated in the resulting tumours[97]. It will be important to examine whether such co-operation exists in human bladder cancer, and the relationship to gender.

The importance of hedgehog signalling in MIBC development has been demonstrated in mouse models and human bladder cancer[99-101]. In mice, urothelial regeneration following injury is driven by sonic hedgehog (SHH)-expressing basal cells, which elicit secretion of factors including the transcription factor GLI1 and WNT pathway proteins by stromal cells, which in turn stimulate proliferation and differentiation of urothelial cells[99]. These cells were also the progenitors of MIBC in the N-butyl-N-4-hydroxybutyl nitrosamine (BBN)-induced urothelial cancer model[100]. Although these progenitors and normal human urothelium express SHH, expression is lost in BBN-MIBC in mice and in human MIBC cell lines[102] and tissues[101]. Dissection of BBN-induced tumorigenesis has revealed that loss of hedgehog signalling blocks production of stromal factors that induce urothelial differentiation, including bone morphogenetic protein 4 (BMP4) and BMP5, suggesting that CIS progression to MIBC is triggered by loss of hedgehog signalling. Importantly, tumour progression could be blocked by pharmacological activation of the BMP pathway,

suggesting a possible therapeutic approach for human NMIBC[101]. Notably, SHH, BMP4 and BMP5 were shown to be significantly downregulated in mRNA sequencing data from the Cancer Genome Atlas (TCGA) study of MIBC, particularly in the aggressive 'basal' subtype[101].

Cell cycle regulation. Almost every MIBC has defects in genes that encode proteins that control the G1 cell cycle checkpoint. Inactivation of *TP53, RB1* and *CDKN2A* is common (Table 2) and has adverse prognostic importance[103]. Taken together with amplification or overexpression of *MDM2*, p53 function was predicted to be inactivated in 76% of MIBCs[37]. Similarly *RB1* loss is common and amplification and overexpression of E2F3, which is normally repressed by RB1, is associated with *RB1* or p16 loss in MIBC[104].

Cyclin D1 (CCND1) and CCND3 are implicated in NMIBC. *CCND1* (11q13) is amplified in ~ 20% of bladder cancers[105]. High nuclear expression in 33% of stage Ta and T1 tumours was associated with higher proliferative index and reduced disease-free survival. High expression of CCND3 (13%) was also associated with reduced survival in bladder cancer[106]. Upregulated expression of these cyclins may represent a more specific mechanism of inactivation of the G1 checkpoint in NMIBC.

Epigenetic alterations: chromatin modifiers at centre stage. Extensive DNA methylation changes have been reported in bladder cancer, many with clinico-pathological associations[107-115]. A major subtype of MIBC has been identified with high-level promoter hypermethylation associated with smoking pack-years[37]. Comparisons of NMIBC and MIBC reveal distinct patterns of hypomethylation in non-CpG islands in NMIBC and widespread CpG island hypermethylation in MIBC[113, 114]. Whilst hypermethylation in promoters is linked to gene silencing, hypomethylation within gene bodies is usually associated with upregulated expression[115]. In medulloblastoma such regions of hypomethylation are marked by H3K4 trimethylation, a marker of open chromatin[116]. Genome-wide analysis of methylation and repressive histone marks also indicates the importance of both DNA and histone methylation in gene silencing[117]. Genomic regions showing DNA copy number-independent transcriptional deregulation were found to be associated with histone H3K9 and H3K27 methylation and histone H3K9 hypoacetylation rather than DNA methylation, a pattern that was related to a CIS-associated expression signature[118].

Genome sequencing of MIBC has identified mutations in chromatin regulators at higher frequency than in other epithelial cancers[11, 17, 36, 37] (Table 2). 76% of MIBCs had an inactivating mutation in one or more chromatin regulating genes[17]. Lysine-specific demethylase 6A (*KDM6A,* a histone demethylase), mixed lineage leukaemia 2 (*MLL2*; also known as *KMT2D*, a histone methyltransferase) and AT rich interactive domain 1A (*ARID1A,* a component of the SWI/SNF chromatin remodelling complex) are frequently mutated. KDM6A demethylates H3K27, leading to a more open chromatin configuration. MLL2 methylates H3K4, also favouring euchromatin and suggesting transcriptional activation. Thus, loss of function of these genes is predicted to lead to gene silencing. Other genes involved in chromatin modification that are mutated in >5% of samples include histone methyltransferases, *MLL* (also known as

KMT2A), *MLL3* (also known as *KMT2C*), histone acetyltransferases E1A binding protein p300 (*EP300*), CREB binding protein (*CREBBP*), a histone deacetylase, nuclear receptor corepressor 1 (*NCOR1*), chromodomain helicase DNA binding proteins *CHD6* and *CHD7,* and a CREBBP activator, Snf2-related CREBBP activator protein (*SRCAP*)[17, 36]. The predominance of inactivating mutations implicates these as tumour suppressor genes. Although several genes are mutated at low frequency, they may be functionally redundant, which may reduce complexity to fewer phenotypic subgroups. For example mutations in *KDM6A* and *MLL2* were reported to be mutually exclusive[37].

Molecular biomarkers

Detailed discussion of prognostic biomarkers is beyond the scope of this Review. However, the application of molecular biomarkers for non-invasive monitoring of NMIBC is approaching clinical applicability and merits brief discussion. As NMIBCs are poorly detected by urine cytology, *FGFR3* mutation analysis combined with other DNA-based biomarkers provides a useful test for disease monitoring[119-121]. Many studies report detection of bladder cancer by analysis of methylation biomarkers in urine. Examples include several panels of biomarkers used alone[113, 122-124] or in combination with *FGFR3* mutation[125]. The most common event described in bladder cancer to date is point mutation of the telomerase reverse transcriptase (*TERT*) promoter in approximately 80% of tumours, regardless of grade and stage. Mutations are mainly at positions -124bp and -146bp relative to the transcriptional start site, allowing development of specific assays that are suitable for detection in urine[126, 127]. Undoubtedly, when used in combination with other urine biomarkers this will improve sensitivity for the detection of bladder cancer.

Pathogenesis

Cell of origin. The normal human urothelium comprises a layer of basal cells in contact with the basement membrane, several layers of intermediate cells, and a single layer of large, superficial 'umbrella' cells with a specialised apical membrane to accommodate bladder expansion and contraction[128]. Lineage tracing studies in mice using SHH-Cre have identified SHH-positive (SHH^+) basal cells that can repopulate the entire urothelium following injury[99]. Whether this is the case in humans is unknown but the presence of large (up to 4.7mm) monoclonal areas within the urothelium comprising basal, intermediate and superficial cell layers, suggests origin from single basal stem cells[129].

Isolation of human bladder cancer stem cells (or tumour-initiating cells) has been achieved using a range of assays (reviewed in[130, 131]). These show features of basal cells residing at the tumour—stromal interface[132]. $CD44^+$ cytokeratin 5 $(KRT5)^+$ $KRT20^-$ tumour cells had enhanced tumour-initiating ability compared with $CD44^-$ $KRT5^-$ $KRT20^+$ cells and could give rise to tumours containing both $CD44^+$ and $CD44^-$ cells in mice[133]. As discussed above, lineage-tracing studies identify SHH^+ basal cells as tumour-initiating cells in mouse BBN-induced invasive tumours[100]. Recent evidence suggests that such cancer stem cells may contribute to therapeutic resistance, by repopulating residual tumours between chemotherapy cycles[134].

In humans, there is evidence for non-basal tumour-initiating cells in more differentiated bladder cancers. Marker combinations corresponding to different urothelial differentiation states could stratify bladder cancer into clinically relevant subgroups, with tumours with the least differentiated (basal) tumour-initiating cells having the worst outcome[135]. Similar evidence was reported from a lineage tracing study in mice, where cells in the intermediate layer were implicated as the cell-of-origin for BBN-induced papillary tumours[136]. Diversity in cancer stem cell phenotype may contribute to the divergent development of NMIBC versus MIBC and in turn define the genomic events that subsequently participate in tumour development.

Molecular features of preneoplastic urothelium. Chromosome 9 LOH is found in 'normal' urothelium and hyperplasia in NMIBC-bearing patients[137-140], and at higher frequency than *FGFR3* mutation in flat hyperplasia, implying that chromosome 9 loss is an earlier event[141]. Dysplasia and CIS are considered precursors of MIBC. These show chromosome 9 LOH, *TP53* mutation[142-144] and multiple other chromosomal alterations[145]. No *FGFR3* mutations are reported in CIS[146]. In morphologically 'normal' or dysplastic urothelium adjacent to MIBC, alterations found in the tumour are present, indicating spread of cells from the tumour or tumour development within a field of altered urothelium[147-150]. Detailed mapping of entire cystectomy specimens reveals that in the absence of detectable abnormality, large macroscopically 'normal' urothelial patches show LOH of specific chromosomal regions. Areas of mild, moderate or severe dysplasia show complex patterns of LOH, suggesting sequential evolutionary 'waves' of change associated with acquisition of growth advantage. Thus, it is envisaged that the development of MIBC involves clonal expansion, within which sub-clones with additional alterations arise (Figure 4). Six critical regions of LOH were identified within one of which, biallelic inactivation of integral membrane protein 2B (*ITM2B)* and lysophosphatidic acid receptor 6 (*LPAR6*, also known as *P2RY5)* were demonstrated[149, 151].

Multifocality, clonality and chronology of events during urothelial carcinoma pathogenesis. The development of multiple bladder cancers in the same patient is common, enabling examination of clonality and molecular evolution. Two concepts have been proposed to explain the origin of multifocal bladder cancer. As a result of extensive carcinogenic insults, many cells may become altered and give rise to independent tumours. Alternatively, a single clone may spread via intraepithelial migration or implantation. Whilst a few patients develop more than one apparently independent tumour (oligoclonal disease)[152], tumours from the same patient are commonly related[153], with evidence for sub-clonal genomic evolution, sometimes complex, in different lesions (reviewed in[154]). Loss of the same chromosome 9 allele in all related tumours again indicates that this is an early event[155-157]. Construction of phylogenetic trees from multiple tumours suggests that $9q^-$, $9p^-$ and $11p^-$ occur early in NMIBC with $8p^-$, $20q^+$, $17p^-$ and $11q^-$ as later events. Interestingly, tumours with the highest genomic complexity are not necessarily the last to appear[156, 158, 159], explaining the observation that recurrent tumours may be of lower grade than preceding tumours[160]. These observations provide further evidence for widespread 'field change' in the diseased bladder. The molecular complexity of related tumours suggests that considerable intratumour heterogeneity may be a feature of

individual bladder cancers, although this has not yet been systematically investigated. Analyses to date on bulk tumour samples may not reveal such heterogeneity. Deep sequencing should now allow such complexity and phylogeny to be examined in more detail.

EMT and metastasis. EMT is a reversible process that involves changes in cell morphology, differentiation and motility, facilitating invasion and metastasis. Markers of EMT (loss of expression of E-cadherin and tight junction proteins and upregulation of vimentin and fibronectin) are associated with MIBC, resistance to therapeutic agents and poor outcome.

EMT is mediated by zinc finger E-box binding homeobox 1 (ZEB1), ZEB2, TWIST, SNAIL (also known as SNAI1) and SLUG (also known as SNAI2), which transcriptionally repress epithelial markers. ZEB1 and ZEB2 are regulated by members of the miR-200 family and miR-205[161]. miR-200 is downregulated in bladder cancer cell lines with mesenchymal phenotype and epigenetic silencing is reported in MIBC[162]. These miRNAs also regulate ERBB receptor feedback inhibitor 1 (*ERRFI1*). Their silencing confers resistance to EGFR in mesenchymal bladder cancer cells that can be reversed by the expression of miR-200[163].

As indicated above, FGFR1 signalling and subsequent COX2 upregulation can induce EMT in bladder cancer[68]. In an animal model of bladder cancer metastasis using an FGFR1-dependent cell line, FGFR inhibition reduced the development of circulating tumour cells and metastasis but not primary tumour growth[69]. Interestingly, although FGFR2 shows reduced expression in MI disease[164], cells selected for enhanced metastatic capability were found to be dependent on FGFR2 isoform IIIc for this phenotype, which was associated with a mesenchymal-epithelial transition (MET)[165]. Thus, FGFRs are implicated in both the EMT required early in the process of metastasis and in the MET required to recapitulate the epithelial phenotype at the metastatic site.

Other factors implicated in bladder cancer cell EMT include the transcription factor inhibitor of DNA binding 1 (ID1)[166] and the long non-coding RNA (lncRNA) H19 (which is upregulated in MIBC), which promotes WNT-β-catenin activation via association with enhancer of zeste homologue 2 (EZH2) and subsequent downregulation of E-cadherin[167]. Similarly, the lncRNA Malat1, which is upregulated by transforming growth factor-β (TGFβ) in bladder cancer cells, induces EMT[168]. *TP63* (which encodes p63) is expressed in the basal and intermediate cell layers of the normal urothelium. Studies of *TP63* isoforms show that ΔNp63 expression is linked to EMT in tumours[169] and this is associated with a more 'basal' phenotype with adverse prognosis[169-171]. Recent expression profiling of large numbers of MIBC defines a claudin-low basal subtype (discussed below) that is characterised by the enrichment of EMT markers and the expression of low levels of cytokeratins[37, 172].

Metastasis of NMIBC is rare, but half of all MIBCs metastasize. Cisplatin-based neoadjuvant chemotherapy prior to cystectomy confers a small survival benefit, believed to be due to the elimination of occult metastases, but survival is poor and improved therapeutic approaches are urgently needed. In addition to

genes involved in EMT, preclinical studies suggest that RHO-GDP dissociation inhibitor 2 (RHOGDI2), the activity of which is regulated by SRC, is a metastasis suppressor in MIBC[173 174]. In contrast to most tumour types, the expression and activation of SRC is highest in NMIBC[174-176], compatible with the activity of RHOGDI2 in these tumours. Further work on the consequences of loss of RHOGDI2 expression implicated endothelin 1 (EDN1) and versican (VCAN) as promoters of an inflammatory environment involving macrophages and the chemokine (C-C motif) ligand 2 (CCL2)-chemokine (C-C motif) receptor 2 (CCR2) signalling axis that is permissive for metastasis[177 178]. A similar relationship of loss of secreted protein acidic and rich in cysteine (SPARC) with metastasis and regulation of inflammatory response in bladder cancer has been reported[179]. Reduced expression of activating transcription factor 3 (ATF3), also a regulator of inflammation, has been implicated in driving metastasis through transcriptional regulation of gelsolin-mediated actin re-modelling. Both proteins are downregulated in MIBC and ATF3 was shown to reduce metastasis in an in vivo experimental model[180]. RAL GTPases RALA and RALB have also been implicated in bladder cancer metastasis[181 182]. CD24, a RAL GTPase-regulated glycosyl phosphatidylinositol (GPI)-linked sialoglycoprotein, is required for metastatic bladder cancer colonisation in mice and shows increased expression in human bladder cancer metastases[183]. Interestingly, prognostic value seems to be confined to male patients and in Cd24-null mice, reduced carcinogen-induced tumour incidence was seen only in males[184]. As CD24 is androgen-responsive, if these data are confirmed, androgen ablation could have therapeutic impact in males[184].

Beyond the 'two pathway' model

The two tumour groupings that have dominated the bladder cancer literature cannot explain the considerable heterogeneity in clinical behaviour. Figure 5 depicts the 'two pathway' model expanded to include potential pathogenic links that are suggested by molecular data. Previous gene-expression profiling studies have reported signatures associated with stage, grade and outcome[185-190]. Recent studies now begin to unravel the heterogeneity in clinical behaviour, revealing multiple molecular subtypes that cut across grade and stage groupings.

mRNA expression analysis of bladder cancers of all grades and stages by Sjödahl *et al.* identified five major subtypes, termed urobasal A (UroA), UroB, genomically unstable (GU), squamous cell carcinoma-like (SCCL) and 'infiltrated'[191], the latter being highly infiltrated with non-tumour cells (Figure 6). Subsequently it was suggested that the SCCL group should be termed 'basal' as it shares features with basal-type breast cancers[192]. UroA and UroB tumours express FGFR3, CCND1 and p63, GU tumours express low levels of these proteins but high levels of ERBB2 and E-cadherin, and SCCL/basal tumours express EGFR, P-cadherin, KRT5, KRT6, KRT14 and proteins involved in keratinisation. UroA tumours showed good prognosis, GU and infiltrated bladder cancers showed intermediate prognosis and SCCL/basal and UroB tumours had the worst prognosis. Whilst UroB have *TP53* mutation and many were MIBC, they show epithelial characteristics including *FGFR3* mutation, which may indicate evolution from UroA tumours.

They also show homozygous deletion of *CDKN2A*, which as discussed above, could be a mechanism by which *FGFR3*-mutant NMIBCs progress[31].

Three subsequent studies of MIBC have defined transcriptional subtypes[37, 172, 193]. There is considerable overlap in these subtypes[194] with two major 'basal' and 'luminal' subtypes that show similarities to intrinsic breast cancer subtypes (Figure 6). Luminal MIBCs are enriched for uroplakins, KRT20, ERBB2 and differentiation markers (forkhead box A1 (FOXA1; also known as HNF3α), GATA binding protein 3 (GATA3), tripartite motif-containing protein 24 (TRIM24) and peroxisome proliferator-activated receptor-γ (PPARγ)), and frequently have papillary morphology and *FGFR3* upregulation and/or mutation. TCGA study defined 4 expression clusters (I-IV), one of which (Cluster I) expressed luminal markers[37]. Choi *et al.*[193] described a luminal subtype termed 'p53-like' with an activated wild-type p53 signature, low levels of cell cycle and proliferation markers, and enrichment of myofibroblast and extracellular matrix markers, perhaps reflecting stromal and fibroblast infiltration[194]. The 'infiltrated' subgroup of Sjödahl *et al.*[191] mostly overlapped with this 'p53-like' subgroup. Tumours in TCGA Cluster II shared features with luminal and p53-like subtypes, and some basal and luminal tumours described by Damrauer *et al.*[172] also exhibited characteristics of the p53-like subgroup. Tumours expressing basal markers (KRT5, KRT6, KRT14, CD44, CDH3) were present in all three studies. Two studies contained claudin-low basal tumours expressing markers that are characteristic of EMT, and low levels of cytokeratins, analogous to some breast cancers[37, 172]. Some 'infiltrated' tumours also showed overlap with this subgroup. In all studies the squamous/basal-type MIBCs showed the worst prognosis and those with papillary architecture, high FGFR3, E-cadherin, GATA3, FOXA1 and uroplakin expression had the best prognosis (Figure 6). Bioinformatics analyses implicated transcription factors that are active in the basal/stem cell compartment of the normal urothelium (signal transducer and activator of transcription 3 (STAT3), nuclear factor-κB (NF-κB), hypoxia-inducible factor 1 (HIF1) and TP63) as potential regulators in basal tumours and PPARγ and oestrogen receptor pathways in luminal tumours[193]. A recent pan-cancer molecular classification also defined a squamous subtype that contains four different tumour types including a subset of TCGA MIBC samples that show worse overall survival than other bladder cancers[195]. Network analysis revealed significant *TP63* expression that was associated with a high level of expression of the oncogenic ΔNp63 isoform. It is likely that squamous/basal MIBCs expressing this isoform represent the lethal subset of p63-expressing advanced bladder cancers that were reported by others[169]. The relevance of p63 expression in poor prognosis UroB tumours remains unresolved, but the expression of different p63 isoforms in NMIBC and MIBC may account for this[169]. It is currently unclear whether analyses that have assessed MIBC only have revealed all biologically relevant heterogeneity as some data suggest that transcriptional subtypes are independent of conventional grade and stage groupings[191]. The reader is referred to an ongoing debate on how to integrate and explore this complexity[196, 197].

Recent studies also indicate potential predictive value. Activation of the EGFR pathway has been identified in basal-type MIBC and it was shown that cell lines with this signature were sensitive to EGFR inhibition[198]. Additionally, the p53-like subgroup of Choi *et al.*[193], contained tumours with resistance to cisplatin-based

chemotherapy and it was confirmed in independent samples and bladder cancer cell lines that this signature had predictive value. Whilst the signature did not identify all resistant tumours, as half of basal-type tumours were also resistant, this represents an important step towards more rational selection of therapy.

Further work will be needed to generate consensus classifiers and robust assays that are suitable for clinical implementation. A simple classifier based on two histology variables (grade and urothelial differentiation pattern) and the expression of two proteins (KRT5, CCNB1) has been reported, which reproduced an original genome-wide expression classification[191] with an accuracy of 0.88[192]. A notable finding was that UroA and UroB tumours retain expression pattern and histology reminiscent of the normal urothelium, with expression of several markers, including proliferative biomarkers in basal cells only, implying retention of dependence on stromal interactions. SCCL/basal tumours had lost this pattern, showing CDH3, KRT5 and EGFR at higher level throughout the tumour parenchyma. This subtype showed a keratin expression profile ($KRT14^+$ $KRT5^+$ $KRT20^-$) that characterises the least differentiated class of tumour initiating cells described by Chan *et al.*[133] and termed 'basal' in the study of Volkmer *et al.*[135].

DNA-based genome-wide analyses also indicate the existence of multiple sub-classes of bladder cancer. DNA copy number and mutation status has identified multiple genomic subtypes of tumours within the conventional grade and stage groupings[43] and MIBCs have been subdivided into three major groups according to copy number alterations and mutation status[37, 43]. The latter groups separate tumours with frequent *TP53* mutation from those with *FGFR3* mutation and *CDKN2A* loss and define a group with an increased frequency of focal amplification and mutations in *MLL2*[37]. DNA methylation profiles also identify bladder cancer subtypes[37, 114, 115, 199]. Four "epitypes"[115] showed broad alignment with previously defined expression subtypes[191, 200]. A key feature of these epitypes was differential expression of homeobox (HOX) genes, with repression of several HOX genes that are also repressed in pluripotent cells in the most aggressive epitype (type D).

A major goal is to integrate information from all platforms to provide the best prognostic and predictive biomarkers for clinical application. As DNA and miRNAs are more robust molecules than mRNA in formalin-fixed paraffin-embedded (FFPE) specimens, a combination of these and protein biomarkers may ultimately provide the most useful classifiers.

Conclusions and future perspectives

For many years molecular understanding of bladder cancer biology has lagged behind that of other solid cancers and this has represented a major barrier to improving clinical care. Although the long-described disease subtypes (NMIBC and MIBC) are clearly distinct at the molecular level, this has not provided adequate prognostic or predictive information for clinical application. With the advent of large-scale genome-wide profiling studies, the field is now poised to replace the conventional 'two pathway' model of bladder cancer pathogenesis with a more complex and molecularly credible description of disease pathogenesis and clinical behaviour and to meet the clinical needs of patients in a more personalised way.

Although non-invasive disease is not life-threatening, its recurrent nature makes this more expensive to treat than other cancers and disease monitoring and treatment are associated with considerable morbidity. Already key biomarker panels hold potential for application in non-invasive urine-based monitoring. A remaining challenge is to develop therapies that can better treat localised disease. Knowledge of the widespread fields of altered cells present in many patients argues for early therapeutic intervention and enhanced efforts to clarify what are the common and targetable early events in NMIBC. Few NMIBCs have been studied by exome or whole genome sequencing and it will be important to establish detailed mutation profiles for this important patient population.

For MIBC, molecular profiling in the context of large clinical trials is required to confirm the ability of molecular signatures to stratify patients prior to treatment with conventional chemotherapy. For these patients, recent insights should also guide the design of new clinical trials, with many possibilities for therapy with targeted agents. The striking finding of frequent somatic mutation of epigenetic modifiers in bladder cancer may offer completely new treatment avenues, where the potential reversibility of phenotype may hold promise for the treatment of some tumour subtypes. Stage T1 tumours are a particularly difficult group to manage. Progression to invasion is relatively common but a major tension exists in considering whether to remove or preserve the bladder. Evidence from studies that include some T1 tumours indicates that as for MIBC, these may be sub-classified to provide objective guidance for treatment. Large collaborative studies are now needed to allow identification of robust prognostic biomarkers and for all subtypes robust diagnostic assays are needed.

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BOX1. Key features of bladder cancer

- Age is the most significant risk factor for bladder cancer, with median age at diagnosis being ~70 years. Bladder cancer is more common in men than women (male:female ratio is 3:1) and it is more common in developed countries.
- In the majority of cases there is no obvious familial history. However, polymorphisms in two carcinogen detoxifying genes, N-acetyltransferase 2 (*NAT2*) and glutathione S-transferase-μ1 (*GSTM1*), confer increased risk and genome-wide association studies have identified single nucleotide polymorphisms (SNPs) that are associated with moderate risk of bladder cancer with odds ratios between 1.1-1.5. Candidate genes close to these SNPs include *MYC, TP63,* prostate stem cell antigen (*PSCA*), telomerase reverse transcriptase (*TERT*)-*CLPTM1L,* fibroblast growth factor receptor 3 (*FGFR3*), transforming, acidic coiled-coil containing protein 3 (*TACC3*), *NAT2,* chromobox homologue 6 (*CBX6*), apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3A (*APOBEC3A*), cyclin E1 (*CCNE1*) and UDP glucuronosyltransferase 1 family, polypeptide A complex locus (*UGT1A*).
- Environmental risk factors include tobacco smoking, occupational exposure to aromatic amines and polycyclic hydrocarbons, consumption of arsenic-contaminated water, chronic infection with *Schistosoma* species, exposure to ionizing radiation and therapeutic abuse of phenacetin-containing analgesics. Cigarette smoking is the major environmental risk factor. It is estimated that more than 50% of cases of bladder cancer can be attributed to smoking.
- Although men are more likely to develop bladder cancer, women often present with more advanced disease and have less favourable prognosis. The reasons for this are not clear.
- In Western countries, more than 90% of cases are urothelial carcinoma. Other histological types include squamous, adenocarcinoma, micropapillary, small cell and plasmacytoid. In regions where the incidence of schistosomiasis is high, squamous cell carcinoma is more common and bladder cancer may be the cancer of highest incidence.
- Most patients present with haematuria and diagnosis is made following cystoscopy and biopsy.
- Treatment of non-muscle-invasive bladder cancer (NMIBC) tumours involves transurethral resection. Surgery may be followed by instillation of a chemotherapy agent, for example, mitomycin C, which delays disease recurrence. This is followed by periodic cystoscopy and cytological

examination of the urine sediment. Where multiple and/or high-grade tumours or carcinoma *in situ* (CIS) is detected, a course of Bacillus Calmette-Guerin (BCG) instillation may be used.

- Many urine-based tests have been proposed to allow non-invasive follow-up. Many tests show greater sensitivity and specificity for detection of muscle-invasive bladder cancer (MIBC) than NMIBC and whilst some are approved for use in the clinic, none have routinely replaced cystoscopic surveillance.
- Organ-confined MIBCs are treated by radical cystectomy or radiotherapy. Chemotherapy may be given prior to or after cystectomy. Five-year survival for patients in this group is approximately 50%. 50% develop metastatic disease and for these median survival is 12-15 months. Cisplatin-based chemotherapy is used but 5 year survival is only ~5%.
- Because of the need for lifetime surveillance and repeated treatment of recurrent disease, bladder cancer poses a considerable economic burden and predicted lifetime costs per patient are higher than those for other cancer types.

Figure legends

Figure 1. Bladder cancer grading and staging.

a. Staging of bladder cancer according to the TNM system[3].

b. Grading according to the 1973 World Health Organisation (WHO) and 2004 WHO/International Society of Urological Pathology (ISUP) criteria[4, 5]. The major difference is in the classification of papillary tumours which are classified as grade 1, 2 and 3 in the older system and as papillary urothelial malignancy of low malignant potential (PUNLMP; equivalent to grade 1), low-grade papillary urothelial carcinoma or high grade papillary urothelial carcinoma in the WHO/ISUP 2004 classification.

Figure 2. The PI3K and MAPK pathways in bladder cancer

Growth factor mediated signalling or mutational activation of both PI3K and MAPK pathways is common in bladder cancer. Receptor tyrosine kinases (RTKs), epidermal growth factor receptor (EGFR), ERBB2, ERBB3, fibroblast growth factor receptor 1 (FGFR1) and FGFR3 may be activated by ligand, over-expression and/or mutation in bladder cancer. Through adaptor proteins, these RTKs activate RAS. Signalling via the RAS-RAF-MEK-ERK cascade leads to phosphorylation of many substrates that can have multiple cellular effects depending on the intensity and duration of signalling. In many situations proliferation is induced. Activated RTKs bind p85, the regulatory subunit of PI3K and recruit the enzyme to the membrane where it phosphorylates phosphatidyinositol-4,5-bisphosphate (PIP2) to generate PIP3. Activated RAS can also directly activate PI3K. PIP3 recruits 3-phosphoinositide-dependent protein kinase 1 **(**PDK1; also known as PDPK1) and AKT resulting in activation of AKT by phosphorylation leading to both

positive and negative regulation of a wide range of target proteins (not all shown). Cyclin D1 (CCND1) and MDM2 are up-regulated directly or indirectly, resulting in a positive stimulus via the RB or p53 pathways, respectively. AKT also phosphorylates and inactivates tuberin, the *TSC2* gene product, leading to activation of mTOR complex 1 (TORC1), which controls protein synthesis. The *TSC1* product hamartin forms an active complex with tuberin, and loss of function of either protein leads to dysregulated mTOR signalling. AKT phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β), relieving its suppression of β-catenin which is freed to enter the nucleus and activate gene expression. MYC expression is induced as a consequence of both by ERK and AKT signalling. Key genes that are activated in bladder cancer are shown in red and those that are inactivated in green. ASK1, apoptosis signal-regulating kinase 1; BAD, BCL-2-associated agonist of cell death; CREB, Cyclic AMP-responsive element-binding protein; FOXO, forkhead box O; IKK, inhibitor of nuclear factor-κB kinase.

Figure 3. Schematic of FGFR3 protein and corresponding exonic positions.

a Codons showing activating point mutation and relative frequencies as percentage of mutations reported in the literature are indicated. SP, signal peptide; IgI, IgII and IgIII, immunoglobulin-like domains; AB, acid box; TM, transmembrane region; TK, tyrosine kinase.

b. Mechanisms of activation of fibroblast growth factor receptor 1 (FGFR1) and FGFR3 in bladder cancer. The receptor-based mechanisms depicted have all been reported in urothelial cancer. There is less information about FGF secretion by urothelial tumour stroma or cancer cells but FGF-like activity is increased in the urine of tumour-bearing patients.

Figure 4. Preneoplastic urothelial alterations in the tumour-bearing bladder

- **a.** What the surgeon sees. At presentation the primary tumour and an area of inflamed urothelium subsequently diagnosed as carcinoma *in situ* (CIS) are resected. During surveillance a second tumour and associated area of CIS are visually identified and resected.
- **b.** The molecular picture. Large areas of urothelium have been replaced with altered cells. Different molecular events are indicated by letters. The primary tumour has developed within a large area of urothelium of normal appearance containing molecular alteration "c". Within this 'field' of change a dysplastic area, also of normal appearance at cystoscopy represents a subclone with 'c+d' and within this, a patch of CIS representing the immediate precursor to the overt tumour (containing c+d+e+f') is recognised as an inflamed patch ('c+d+e'; shown in blue). A second tumour and CIS resected at a later date have also developed within the large altered field containing 'c' but differ in all other alterations. The possibility that tumours with independent initiating events may develop in some patients is indicated by patches 'a' and 'b', both of which may remain morphologically silent for many years. The dotted line indicates a urothelial patch as described by Gaisa *et al.*[123] and putatively derived from a single stem cell. Despite independent tumour-initiating events, cells with 'a' and 'b'

are clonally related. The presence of large and clinically silent areas of preneoplastic urothelium emphasises the need for novel approaches for local therapeutic targeting.

Figure 5. Potential pathways of urothelial carcinoma pathogenesis

Potential pathogenesis pathways are shown based on histopathological and molecular observations. The blue and purple pathways indicate the two major pathways with distinct histopathological and molecular features that have been recognised for the past two decades. Percentages indicate percentage at diagnosis. Key molecular features are indicated. The recent identification of multiple distinct molecular subtypes of non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) suggests multiple sub-pathways within each of the major pathways, as shown. It should be noted that the existence of multiple subtypes within all of the histologically recognisable intermediates remains hypothetical. Development of histopathologically recognisable urothelial alterations is preceded by clonal expansion of altered cells within the urothelium. Low-grade papillary tumours may arise via simple hyperplasia and minimal dysplasia and these are characterised at the molecular level by deletions of chromosome 9 and activating mutations of fibroblast growth factor receptor 3 (*FGFR3*)*, PIK3CA* and stromal antigen 2 (*STAG2*). These non-invasive tumours recur frequently but are genetically stable. Invasive carcinoma is believed to arise via flat dysplasia and carcinoma *in situ* (CIS), which commonly show *TP53* mutation in addition to chromosome 9 deletions but no *FGFR3* mutations. Invasive tumours are genetically unstable and accumulate many genomic alterations. Uncertainty about the possible development of high-grade non-invasive papillary tumours from flat dysplasia is indicated by a dashed arrow. The finding of a subtype of invasive carcinomas with *FGFR3* mutation and loss of *CDKN2A* may suggest a route by which low-grade non-invasive papillary tumours can progress to muscle invasion (dashed arrow).

Figure 6. Molecular subtypes of bladder cancer

Subtype classification according to the mRNA expression profiling studies. **a**. Sjödahl *et al.*[164] ; **b**. Damrauer *et al.*[182]; **c**. The Cancer Genome Atlas (TCGA) study[36]; **d**. Choi *et al.*[183]. Subtype designation is indicated at the top of each figure. Heatmaps show representative expression levels of selected genes that are characteristic of the molecular subtypes defined in each study. Red, high expression; light green, low expression; dark green, intermediate expression, heterogeneous expression, or expression only in certain layers of the urothelium. Mutation status, copy number alterations, histological characteristics and information relating to prognosis and treatment response are shown where such information is available. Black boxes indicate the presence of a mutation, copy number alteration or histological feature. *CCND1*, cyclin D1; DSS, disease-specific survival; *EGFR*, epidermal growth factor receptor; *FGFR3*, fibroblast growth factor receptor 3; *FOXA1*, forkhead box A1; KRT, cytokeratin; MI, muscle-invasive; NAC, neoadjuvant chemotherapy; OS, overall survival; PPARG, peroxisome proliferator-activated receptor-γ; *TSC1*, tuberous sclerosis complex 1; UPK, uroplakin.

Table 1. Oncogenes altered in bladder cancer

Gene	Chromosome	Alteration in low-grade stage Ta	Frequency	Alteration in muscle-invasive (≥T2)	Frequency	Function
NRAS	1p13	Point mutation	1-2%	Point mutation	1-2%	Cytoplasmic GTPase
PIK3CA	3q26	Point mutation	25%	Point mutation	9-20%	PI3K catalytic subunit-α (also known as p110α)
FGFR3	4p16	Point mutation Upregulated expression	60-70% 80% (protein)	Point mutation Upregulated expression	5-20% 40% (protein)	Protein tyrosine kinase growth factor receptor
E2F3	6p22	Amplification Upregulated expression	0% 10% (protein)	Amplification Upregulated expression	9-14% 20% (protein)	Transcription factor; cell cycle control; RB binding activity
EGFR	7p12	Upregulated expression	~20% (protein)	Upregulated expression	~50% (protein)	Protein tyrosine kinase growth factor receptor
FGFR1	8p12	Amplification Upregulated expression	1% ~50% (mRNA)	Amplification Upregulated expression and isoform switching	6% ~80% (mRNA)	Protein tyrosine kinase growth factor receptor
*RXRA**	9q34	NI	NI	Mutation	9%	Retinoic acid receptor; transcription factor
HRAS	11p15	Point mutation	5-10%	Point mutation	5-6%	Cytoplasmic GTPase
CCND1	11q13	Amplification	20%	Amplification	20%	Cyclin
KRAS	12p12	Point mutation	5%	Point mutation	5%	Cytoplasmic GTPase
*ERBB3**	12q13	NI	NI	Point mutation	11%	Protein tyrosine kinase growth factor receptor
MDM2	12q14-q15	Amplification	3%	Amplification	4-5%	E3 ubiquitin ligase; targets p53
AKT1	14q32	Point mutation	1-3%	Point mutation	1-3%	Serine-threonine protein kinase
*ERBB2**	17q12	NI	NI	Amplification Upregulated expression Mutation	5-14%; 42% in micropapillary variant 8-30% (protein) 8%	Protein tyrosine kinase growth factor receptor

*Genes identified as significantly mutated in ≥5% of cases in the largest exome and whole genome sequencing study reported to date[36].

CCND1, cyclin D1; *EGFR*, epidermal growth factor receptor; *FGFR*, fibroblast growth factor receptor; NI, no information; *RXRA*, retinoid X receptor-α

Table 2. Putative tumour suppressor genes with loss of function in bladder cancer

Gene	Chromosome	Alteration in low-grade stage Ta	Frequency	Alteration in muscle-invasive (≥T2)	Frequency	Function

RUNX3	1p36	Promoter hypermethylation Inactivating mutation	65% 1-2%	Promoter hypermethylation Inactivating mutation	85% 1-2%	Runt-domain transcription factor
*ARID1A**	1p35	NI	NI	Inactivating mutation	25%	Component of SWI/SNF chromatin remodelling complex
*TXNIP**	1q21	NI	NI	Inactivating mutation	7%	Thioredoxin interacting protein; redox regulator
*ELF3**	1q32	NI	NI	Inactivating mutation	8%	ETS domain transcription factor; involved in urothelial differentiation
*NFE2L2**	2q31	NI	NI	Inactivating mutation	8%	Transcription factor; regulation of anti-oxidant program
CTNNB1	3p21	Inactivating mutation	~2%	Inactivating mutation	~2%	Adherens junction complex; actin cytoskeleton anchor; component of WNT signalling pathway
*FBXW7**	4q31	NI	NI	Inactivating mutation	10%	F-box family protein; protein ubiquitylation
PIK3R1	5q13	Mutation	5%	Mutation	5%	Regulatory subunit of PI3K (also known as p85
APC	5q21-q22	Inactivating mutation	2-4%	Inactivating mutation	9-16%	Defective in adenomatous polyposis coli; antagonist of WNT signalling and other functions
*CDKN1A**	6p21	NI	NI	Inactivating mutation	14%	p21; cyclin-dependent kinase inhibitor
CDKN2A	9p21	Hemizygous deletion Homozygous deletion Promoter methylation Mutation	50-60% 15% Conflicting data NI	Hemizygous deletion Homozygous deletion Promoter methylation Mutation	50-60% 20-30% Conflicting data 5%*	Encodes p16 and $p14^{ARF}$; cyclin-dependent kinase inhibitor
PTCH1	9q22	Inactivating mutation	NI	Inactivating mutation	7%	Sonic hedgehog receptor
TSC1	9q34	Inactivating mutation	11-16%	Inactivating mutation	11-16%	Negative regulator of mTORC1 signalling in complex with TSC2
PTEN	10q23	Hemizygous deletion Homozygous deletion	6-8% 0%	Hemizygous deletion Homozygous deletion	25-58% 4-6%	Protein and lipid phosphatase; negative regulation of AKT signaling
*ATM**	11q22-23	NI	NI	Inactivating mutation	14%	Cell cycle checkpoint kinase
*MLL2**	12q13	NI	NI	Inactivating mutation	27%	Histone methyl transferase
MDM2	12q14-q15	Amplification	3%	Amplification	4-5%	E3 ubiquitin ligase; targets p53
RB1	13q14	Inactivating mutation	NI	Inactivating mutation	11% - 13%	Negative regulator of the cell cycle; heterochromatin stability; tumour suppressor
*KLF5**	13q22	NI	NI	Inactivating mutation	8%	Kruppel-like family transcription factor
TSC2	16p13	Inactivating mutation	2%	Inactivating mutation	2%	Negative regulator of mTORC1 signalling in complex with TSC1

TP53	17p13	Inactivating mutation Upregulated expression	0-14% ≤10%	Inactivating mutation Upregulated expression	24-56% 30-50%	Transcription factor; cell cycle & stress response; induces apoptosis
*ERCC2**	19q13	NI	NI	Inactivating mutation	12%	Defective in xeroderma pigmentosa complementation group D; functions in nucleotide excision repair
*EP300**	22q13	NI	NI	Inactivating mutation	15%	Histone acetyltransferase; transcriptional co-activator
*KDM6A**	Xp11	NA	NA	Inactivating mutation	24%	Histone demethylase
STAG2	Xq25	Inactivating mutation	32-36%	Inactivating mutation	9-13%	Cohesin complex; DNA repair; transcriptional control

*Genes identified as significantly mutated in ≥5% of cases in the largest exome and whole genome sequencing study reported to date[36].

ARID1A, AT rich interactive domain 1A; *ATM*, ataxia-telangiectasia mutated; *CDKN*, cyclin-dependent kinase inhibitor; *CTNNB1*, β-catenin; *ELF3*, E74-like factor 3; *EP300*, E1A binding protein p300; *FBXW7*, F-box and WD repeat domain containing 7; *KDM6A*, lysine-specific demethylase 6A; *KLF5*, Kruppel-like factor 5; KMT2D, lysine-specific methyltransferase 2D; *MLL2*, mixed lineage leukaemia; mTORC1, mTOR complex 1; *NFE2L2,* nuclear factor erythroid 2-like 2; NI, no information; *PTCH1,* patched 1; RUNX, runt-related transcription factor; *STAG2*, stromal antigen 2; TSC, tuberous sclerosis complex; *TXNIP*, thioredoxin interacting protein.

Glossary

TNM classification system: This system of classification is used to describe the stage of a tumour. T describes the extent of local invasion; N describes whether the tumour has spread to local lymph nodes; M describes distant metastatic spread.

BCG therapy: Bacillus Calmette-Guerin is a vaccine used to induce immunity to tuberculosis. It is also used to treat high-risk localised bladder cancer and carcinoma *in situ*. Instillation into the bladder induces a localised immune response that is able to eliminate cancer cells. For bladder cancer treatment, a course of 6 weekly treatments is usual

Chromothripsis: Chromosomal shattering. A phenomenon whereby cancer cells acquire many clustered chromosomal rearrangements as a single catastophic event during tumour development.

Non-homologous end joining: An error-prone mechanism of DNA repair where broken DNA ends are joined without the guidance of a large homologous template. Short homologous DNA sequences termed microhomologies are used to guide repair.

Replication-licensing complex: A complex of proteins that assembles at origins of DNA replication during late G1 phase of the cell cycle to ensure precise and timely DNA replication.

Homologous recombination: A high fidelity mechanism of DNA repair that uses recombination with an intact homologous template to repair double strand breaks.

Smoking pack-years: A measure of exposure to cigarette smoke that is calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. For example,

1pack-year is equal to smoking 20 cigarettes (1 pack) per day for 1year, or 40 cigarettes per day for half a year.

Euchromatin: Less densely packed or 'open' chromatin that is often associated with active gene transcription.

Field change: A process by which molecular alterations are accumulated within a large tissue area in response to carcinogenic stimuli.

Author biographies

Margaret Knowles received her PhD in 1977 from the University of London, UK where she worked with Dr Sammy Franks at the Imperial Cancer Research Fund Laboratories (now Cancer Research UK London Research Institute) on carcinogen-induced transformation of epithelial cells. After Postdoctoral work on organ culture of human bladder with Drs Marian Hicks and Roger Berry at The Middlesex Hospital Medical School, London she established a group working on the molecular biology of bladder cancer at the Marie Curie Research Institute, Oxted. She moved to the University of Leeds as Professor of Experimental Cancer Research in 1997 where her group continues to focus on the molecular features of bladder cancer.

Carolyn Hurst received her PhD in 1993 from Swansea University, UK where she worked with Professor David Skibinski on the population genetics of marine invertebrates. After finishing her PhD she undertook postdoctoral positions working as a molecular biologist at the Memorial University of Newfoundland, Canada and the University of Greenwich, London, UK. Since 2001 she has worked in Margaret Knowles's group at the University of Leeds, with particular focus on genomics and genome-wide profiling of bladder tumours.

Key points

- Bladder cancer is the fifth most common cancer in men in Western countries (male to female ratio is 3:1) with tobacco smoking as a major risk factor.
- There are two major groups of patients with distinct prognosis and molecular features. Although local disease recurrence is a major problem for those with low-grade non-muscle-invasive tumours, life expectancy is long and development of invasive disease infrequent. For those who present with muscle-invasive disease, development of metastatic disease is common, prognosis is dismal and no advances in therapy have been made for decades.
- Major unmet clinical needs include non-invasive methods for disease surveillance and novel approaches to eliminate both tumour and widespread intraepithelial preneoplasia in patients with non-muscle-invasive disease. New systemic therapeutic approaches are urgently needed for those with muscle-invasive disease. Recent studies reveal important biological features of urothelial metastasis and the epithelial-mesenchymal transition that may contribute to metastatic initiation. A key role for inflammatory processes is evident in the development of metastasis.

- Heterogeneity in outcome within the two major groups indicates a need for sub-classification for more accurate prognostication, prediction of response to current therapies and development of novel therapies.
- Recent molecular analyses now provide such sub-classification with definition of multiple sub-groups that are independent of conventional histopathological definitions. This presents major opportunities for personalised patient care.

ToC blurb

This Review discusses the recent advances in the molecular characterisation of bladder cancer, which has provided insight into pathogenesis and subgroups of bladder cancers, with differing prognosis.

Subject categories

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