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Genomic heterogeneity in bladder cancer: challenges and possible solutions to improve outcomes

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Abstract

Histological and molecular analyses of urothelial carcinoma often reveal intratumoural and intertumoural heterogeneity at the genomic, transcriptional and cellular levels. Despite the clonal initiation of the tumour, progression and metastasis often arise from subclones that can develop naturally or during therapy, resulting in molecular alterations with a heterogeneous distribution. Variant histologies in tumour tissues that have developed distinct morphological characteristics divergent from urothelial carcinoma are extreme examples of tumour heterogeneity. Ultimately, heterogeneity contributes to drug resistance and relapse after therapy, resulting in poor survival outcomes. Mutation profile differences between patients with muscle-invasive and metastatic urothelial cancer (interpatient heterogeneity) probably contribute to variability in response to chemotherapy and immunotherapy as first-line treatments. Heterogeneity can occur on multiple

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Author contributions

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Competing interests

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levels and averaging or normalizing these alterations is crucial for clinical trial and drug design to enable appropriate therapeutic targeting. Identification of the extent of heterogeneity might shape the choice of monotherapy or additional combination treatments to target different drivers and genetic events. Identification of the lethal tumour cell clones is required to improve survival of patients with urothelial carcinoma.

Heterogeneity resulting from clonal expansion of individual mutations, genomic alterations and variability of gene expression between tumour regions and, in patients with metastasis, between the primary tumour and metastases, forms the basis of the complexity of cancer[1]. Tumour heterogeneity is recognized as a hallmark of urothelial carcinoma and is potentially related to high mutational burden that can change cellular differentiation over time with each cell division[2,3]. Next-generation sequencing enables identification and characterization of urothelial carcinoma heterogeneity at the genomic and transcriptomic levels and provides the opportunity to associate alterations with tumour morphology and clinical outcome[4]. Nevertheless, tumour heterogeneity is a considerable obstacle for both scientists and clinicians when developing new agents or choosing therapeutic strategies for treatment of patients with urothelial carcinoma[5]. For example, treatments directed at individual genomic targets are likely to result in expansion of non-responding clones that do not harbour these targets, and less-targeted therapies (for example, chemotherapy and immunotherapy) might fundamentally alter the clonal and/or transcriptional subtypes of an individual tumour[6].

Evidence for the critical role of tumour heterogeneity was identified in lung cancer[7], renal cell carcinoma[8] and colorectal cancer[9,10]. In these detailed studies of multiple tumour sites, parental driver alterations are shared, but new clones result over time. For example, in lung cancer, driver mutations in *TP53, MET, EGFR* and *BRAF* were often clonal, whereas alterations in *PIK3CA, NF1* and DNA damage repair and chromatin-regulatory genes were more heterogeneous and occurred as later alterations[7]. New methods and model systems for assessing and studying the effects of tumour heterogeneity on phenotype and clinical behaviour will facilitate an improved understanding of this cancer hallmark in urothelial carcinoma[11].

Bladder cancer heterogeneity occurs on multiple levels and directly affects clinical care. Patients with bladder cancer usually die from muscle-invasive disease and much research is focused on this disease stage. However, 75% of patients are diagnosed with non-muscle-invasive disease[12] and tumour heterogeneity is likely to have a role in the management of these patients, as it might affect the selection of non-muscle-invasive bladder cancer (NMIBC) risk groups, surveillance monitoring strategies, intravesical therapies and early application of radical therapy[13]. Unfortunately, few data exist to suggest that substantial tumour heterogeneity is present and a driver of treatment resistance in NMIBC. In addition, the total mutation burden reported for NMIBC is lower than that of muscle-invasive bladder cancer (MIBC)[14], and data on the application of tumour subtypes to NMIBC are limited. Overall, tumour heterogeneity affects several major aspects of bladder cancer management: molecular profiling of MIBC to assess risk of relapse, selection of aggressive tumours (NMIBC or MIBC) for radical treatment, and use of urine and blood biomarkers to identify aggressive tumours, apply early radical therapy and identify relapse.

In this Review, we describe the multiple levels of heterogeneity in bladder cancer and how they affect tumour biology and clinical care. We summarize common definitions of tumour heterogeneity and discuss the link between heterogeneity and tumour evolution, as well as the influence of treatments and molecular drivers. We then describe current knowledge of genomic heterogeneity at the DNA level and the expression level, resulting in different tumour subtypes and the morphological heterogeneity seen in variant bladder cancer histology. Finally, we discuss the influence of heterogeneity on treatment decision-making, drug development and clinical trial design.

Definitions of tumour heterogeneity

Improved technology to characterize the heterogeneity of tumours at the morphological, genomic and transcriptional levels led to an appreciation by clinicians that neoplastic disease is inherently unstable, characterized by heterogeneous tumour composition and evolving morphological changes that occur in the disease course. With this recognition, scientists and clinicians have worked to develop a framework to understand the heterogeneity of urothelial carcinoma between tumours, within a tumour and over time (BOX 1; FIG. 1).

Interpatient heterogeneity.

Patients with cancer have traditionally been characterized clinically by the location of their primary tumour and its histology. Clinicians treat eligible patients with metastatic urothelial carcinoma using cisplatin-based chemotherapy; some of these patients will initially respond very well to this therapy and others will not respond at all[3]. Currently, the molecular features associated with response to chemotherapy are not fully understood but might include tumour immune cell invasion (for example, indicated by PDL1 status), total mutation burden (a surrogate for neoantigen load), DNA damage response defects and tumour morphology[6]. As genomic and molecular characterization become routinely utilized, this information can help to evaluate interpatient heterogeneity in patients with the same tumour type, providing new data to characterize patients' tumours beyond their histological information and determine their optimal treatment plan.

Intratumoural heterogeneity.

Perhaps the most commonly referenced type of heterogeneity for urothelial carcinoma is intratumoural heterogeneity, which describes differences among regions of the primary tumour that might have discreet genomic and functional alterations during tumour evolution[7,8,15]. Intratumoural heterogeneity has long been recognized clinically by histological differences in distinct tumour areas. For example, in prostate cancer, the Gleason Score is calculated by adding the two most prominent grades from different tumour areas to give a sum score[16]. Specific clinical considerations for patients with urothelial carcinoma include whether multiple areas of the tumour should be biopsied; how the molecular information might influence care and whether different molecular targets exist; and — when multiple histologies are present in the same tumour — whether the more aggressive histology and its relative representation can be identified.

Intertumoural heterogeneity.

This type of heterogeneity refers to differences between the primary tumour and metastases, different metastatic sites or multiple tumours found in the same primary location. Research from patients with metastases suggests that very few genomic alterations are shared between the primary tumour and the metastases[17]. Several investigations have demonstrated heterogeneous genetic findings of metastatic lesions from the same patient: the genetic makeup of some of the lesions represented that of a subclonal population in the primary tumour, whereas others represented distinct mutations found only in the metastatic lesions[8,18,19]. The key consideration for the clinician is when to biopsy a metastatic deposit and how to use the information gained from genetic analysis. The identification of a new or different mutation in a metastasis might indicate the need to change therapy, add additional therapy or treat a rogue metastatic lesion with local therapy if it is believed to be an isolated event in a patient with otherwise adequate systemic disease control.

Temporal heterogeneity.

Temporal heterogeneity describes changes in the tumour over time. Urothelial carcinomas are inherently genetically unstable and new mutational events occur frequently, which can accumulate over time[20,21]. Temporal heterogeneity is more likely to affect patients with metastatic cancer[22]. One practical consideration for clinicians and when planning a clinical trial is to determine when archival tissue is acceptable or when a new biopsy is needed. Clinically, the answer is sometimes self-evident, for example, in a patient with a tissue sample from many years ago who now has recurrent disease a biopsy is likely to be part of the assessment of new drivers in the metastases. In trials that involve evaluation of genomic alterations, archival tissue for analysis is commonly allowed if available from within a certain time period (for example, from the past 12 months)[23]. Clinicians are hesitant to pursue re-biopsy owing to the potential medical risks, discomfort to the patient and cost[24]. The rapidly expanding availability and utility of blood-based and urine-based liquid biopsies might obviate this problem[25,26].

Circulation heterogeneity.

Circulation heterogeneity refers to differential genomic profiles of circulating DNA compared with tissue from the primary or metastatic site[27,28]. Liquid biopsy is the measurement of the cell-free DNA in the blood[29] but can be extended to include circulating tumour cells[30]. The utility of liquid biopsies is rapidly improving as technological advances are made and they are increasingly used in both clinical and trial settings[31]. Several important questions regarding circulation heterogeneity in urothelial carcinoma currently remain unanswered. For example, whether direct comparison of genomic alterations and allele frequencies between tissue, urine and blood samples is possible; which platforms, tumour types and tumour burdens are best evaluated by blood assays; and whether integrating data from multiple tumour sites with divergent evolution is meaningful. Much work is being done to address these questions and developments in blood-based assays now provide the opportunity for longitudinal and frequent assessment, especially of specific mutational events that can be targeted with therapy at progression[32].

Heterogeneity and branching evolution

Urothelial carcinoma is characterized by a high total mutational burden of >7 mutations per Mb — only exceeded by lung and skin cancer[33]. This high mutation rate is believed to fuel tumour heterogeneity and tumour evolution (FIG. 2). Several studies have examined the evolutionary dynamics of urothelial carcinoma[2,17,34–36]. Mutational analyses of early-late tumour pairs identified the existence of a single ancestral origin within each assessed patient demonstrated by identical mutations at a high cellular prevalence in the primary and invasive tumour pairs. Furthermore, subclonal mutations that were specific to the individual tumours were identified[34]. Branched evolution was also found as an early event in the natural history of urothelial carcinoma with metastasis. Phylogenetic analysis of 21 sets of matched early and late tumours showed that the ancestral clone gave rise to multiple cell populations that evolved in parallel during the early stages of tumour evolution[17]. A high level of intertumoural heterogeneity between primary tumours and metastases was also seen in another study[37]. Evaluation of molecular features of metachronous tumours from 29 patients initially diagnosed with early-stage bladder cancer revealed a common origin of the metachronous tumours that developed years later. Tumours from patients with progressive disease had a higher variation in the intrapatient mutational spectrum and a higher frequency of APOBEC-related mutations than those from patients with non-progressive disease[2]. Genomic studies have shown a significant difference in the number and frequency of individual mutations and rearrangements between ancestral and progressive clones[4]. Frequent mutations of tumour suppressors and oncogenes, including in *KDM6A, TP53, PIK3CA* and *FGFR3*, were found in the ancestral clone, whereas mutations in *TP53, MLL3, FBXW7* and *SETD2* were found in progressing clones[2,34]. Non-aggressive subclones in early tumours harboured mutations (in *FGFR3*, *AFDN* and *H3F3A*) that were absent in invasive clones. Fibroblast growth factor (FGF) signalling was enriched in ancestral clones of early invasive urothelial carcinoma with *PIK3CA* mutations, whereas DNA damage checkpoint regulation signalling was enriched in progressing subpopulations[34]. The differences in genes altered before and after progression suggest that tumour evolution continues as a function of time.

Chemotherapy-driven clonal evolution.

The effect of systemic therapies on the evolutionary trajectory of urothelial carcinoma (FIG. 2) was studied by comparing the genomic profiles of samples from matched untreated and chemotherapy-resistant tumours from individual patients[17]. Whole-exome sequencing and clonality were estimated in tumour analyses of 16 matched chemotherapy-naive and cisplatin-treated tumours. Only one-third of the mutations were shared within the tumour pairs, demonstrating mutational heterogeneity for each pair. Reconstructing the phylogenetic relationship of each patient's samples revealed early branching evolution occurring in successive waves of clonal expansion. The observed increase in the clonality of mutations found in post-chemotherapy tumours suggested that chemotherapy restricted the mutational landscape of the tumour[17]. Chemotherapy-resistant tumours were enriched in genes involved in integrin signalling, which is linked to cell-adhesion-mediated survival and drug resistance[38]. Increased activity of integrin signalling pathways is a possible shared link between drug resistance and metastatic spread of urothelial cells. These findings are

consistent with mathematical models showing that even a small advantage in a single cell under selective pressure from chemotherapy can enable the descendants of this resistant cell to replace the precursor tumour mass, thereby increasing clonality and restricting mutational heterogeneity[39]. A study of the mutational patterns in chemotherapy-resistant muscle-invasive urothelial carcinoma using whole-exome sequencing of matched samples from 30 patients before and after neo-adjuvant cisplatin-based chemotherapy identified a new cisplatin mutation signature, which was linked to 14% of mutations in treated tumours, supporting the idea that chemotherapy shapes the mutational landscape of urothelial carcinoma[6]. The cisplatin mutation signature is enriched in T>A and C>A mutations compared with other mutational signatures, such as the APOBEC (C>T) and mutation signature 5 (comparatively flat signature with minimal signature peaks). Collectively, these data suggest that systemic chemotherapy for urothelial carcinoma affects the evolution of a cancer, constraining the clonality, and ultimately leading to treatment resistance.

APOBEC3-related mutagenesis.

The true initiating steps of bladder cancer are unknown, but the development of genomic mutations is likely to have a fundamental role. Compared with other solid tumours, the high mutational burden in urothelial carcinoma might be partly driven by enzymatic activity. The DNA-editing enzyme apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC3) family consists of seven enzymes[34,40]. These enzymes are members of a super family of evolutionarily conserved deaminases, including activation-induced cytidine deaminase, APOBEC1, APOBEC2 and APOBEC4 (REF.[41]). APOBEC3 enzymes are known for their role in restricting viruses by editing viral DNA[42]. APOBEC3-induced DNA editing is caused by the deamination of cytidines (C) to uridines (U), which are repaired to guanines (G) or thymidines (T)[41]. Each of the seven human APOBEC3 para-logues has a preferred cytidine-harbouring motif. For instance, APOBEC3B preferentially deaminates cytidines in a TCW motif (in which W can be A or T)[43]. APOBEC3-induced mutational signatures are prevalent in bladder, cervical, breast, head and neck, and lung cancers[44]. Analysis of gene expression data and mutation patterns, distributions and loads of 19 different cancer types showed that APOBEC3B-catalysed genomic uracil lesions are responsible for a large proportion of mutations in urothelial carcinoma[45].

The frequency of APOBEC mutational signatures found at all stages of bladder cancer provides evidence for a role of APOBEC in this disease[40]. APOBEC3 mutational signatures become enriched during progression from early-stage to muscle-invasive urothelial tumours[2]. These signatures have been specifically identified in high-risk NMIBC and potentially fuel tumour progression and evolution, even in these early stages[46]. Analysis of the mutational signatures from The Cancer Genome Atlas (TCGA) muscle-invasive urothelial carcinoma patient cohort show that both APOBEC3A and APOBEC3B signatures accounted for 67% of single nucleotide variations[40]. Patients with APOBEC-enriched tumours had a better prognosis (median survival 50 months versus not reached, 1.5×10^{-4})[40]. Whether this improved survival reflects better response to treatment is not known.

APOBEC3A and APOBEC3B expression levels also correlate with APOBEC3-associated mutational load[47]. Several studies suggest that APOBEC3-associated mutations have a role

in shaping urothelial carcinoma evolution. More than 40% of clonal mutations in cancer driver genes of several tumour types, including urothelial carcinoma, were found to have APOBEC-signature enrichment[1]. In urothelial carcinoma, 62% and 75% of mutations associated with APOBEC3A and APOBEC3B are clonal, respectively, suggesting that the majority of APOBEC3 signature mutations occur early in urothelial carcinoma evolution[40]. Mutational signatures associated with APOBEC3A and APOBEC3B were enriched in urothelial carcinoma after cisplatin-based chemotherapy[17]. APOBEC3 activity is enriched in lagging DNA strands in early-replicating, gene-dense and active chromatin regions and it is plausible that conditions that increase the abundance of single-strand DNA, such as chemotherapy, could increase the substrate availability for APOBEC3-induced mutagenesis[43,48–53]. These data suggest a potential interaction between chemotherapy and APOBEC3-induced mutagenesis in shaping the evolutionary landscape of urothelial carcinoma. In addition, previous findings in breast cancer models suggest that APOBEC3B promotes tamoxifen resistance[54]. Whether APOBEC3 enzymes have similar roles in treatment resistance in urothelial cancer remains to be determined.

Gene expression heterogeneity

The substantial intertumoural heterogeneity identified in urothelial carcinoma might be driven by variations in cell cycle activity and cellular differentiation programmes between patients[55]. To classify the differences in gene expression between urothelial carcinomas, a system of molecular subtypes was proposed, with distinct molecular characteristics and associations with pathological findings and clinical outcome[40,46,55]. The characteristics and evolution of subtypes in NMIBC and MIBC have been previously described[13,56] and, in MIBC, a consensus classification of six subtypes is emerging, which includes luminal papillary, luminal non-specific, luminal unstable, stroma-rich, basal squamous, neuronal-like subtypes[57]. Some studies have observed molecular subtypes to be associated with response to therapeutic treatment, but conflicting results have been reported and no consensus exists[58–62].

Interpatient heterogeneity is likely to be caused by a range of underlying DNA changes (mutations, rearrangements, insertions or deletions, long non-coding RNAs and methylations) accumulated during the evolution of each cancer, but observed differences might also be a product of varying cell-type compositions in the analysed tissue sample. The constant evolution of the cancer genome generates new genomic subclones[63] that might give rise to differences in gene expression patterns within the tumour. However, most analyses of molecular subtypes are based on the assumption that no intratumoural heterogeneity exists and try to assign a single subtype to each tumour. Multiple subtype classification studies have reported unclassified samples and varying classification strengths (for example, silhouette width measures[64]), suggesting that substantial intratumoural heterogeneity in gene expression exists. This heterogeneity might also be caused by undiscovered subtypes, but, on the basis of current knowledge of genomic evolution, several subtypes are likely to coexist in single tumours.

Studies of temporal intratumoural heterogeneity have shown differences in tumour classifications. In a study from 2005 of array-based gene expression analysis in

metachronous tumours, most paired tumour samples had similar expression patterns but several exceptions were found[65]. In 9 of 14 patients, tumours at presentation had gene expression similar to tumours at progression. Across all patients, gene expression of early-stage and late-stage tumours from an individual patient was more similar than that of tumours at the same stage across patients. New studies of synchronous and metachronous tumours had similar results[2,46,66]. A study in 57 primary MIBCs and 28 matched lymph node metastases found only 18% discordance (12 of 67 pairs) in subtype classification between primary tumours and metastases overall but 58% discordance (7 of 12 pairs) in the basal/squamous-like subtype[67]. In 6 of these 12 discordant pairs, the primary tumour showed substantial intratumoural heterogeneity, including mesenchymal, genomically unstable and small-cell neuroendocrine subtype regions. Thus, discordance might be caused by intratumoural heterogeneity in half of these pairs and the rest might reflect subtype plasticity.

In a study of spatial intratumoural heterogeneity in four patients[68], the authors identified both luminal-like and basal-like gene expression subtypes in laser-microdissected tumour tissue from cystectomy specimens[68]. Importantly, different subtype classifications were only observed in the two patients with multifocal tumours. In one patient, basal-like expression patterns were observed in a muscle-invasive tumour and luminal-like expression patterns in synchronous Ta tumours. However, in the second patient with multifocal MIBC, the luminal-like and basal-like expression subtypes were intermixed in different areas of the individual tumours. Overall, the gene expression differences mirrored genomic alterations in tumour biopsy samples, suggesting that gene expression patterns might be founded in DNA alterations. In TCGA MIBC data set, associations of mutation patterns specifically with a tumour subtype were limited, although luminal papillary tumours were enriched in FGFR3 alterations[56]. Histone regulation might have a role in subtype development, as luminal tumours have an increased frequency of KDM6A mutations, which are also found in low-grade (papillary) tumours. Thus, subtypes might be affected by genetic and epigenetic mechanisms of regulation.

Collectively, intratumoural heterogeneity complicates gene expression subtyping of a subset of bladder tumours, and average bulk tumour estimates might confound subtype analysis (for example, for therapy response estimation), and more advanced approaches to subtyping might be required. In colorectal cancer, a meta-analysis of expression subtypes documented evidence for a continuous subtype score instead of the traditional subtype association approach[69]. The high mutation rate and intratumoural heterogeneity observed in bladder cancer suggests that differences in gene expression patterns are likely to arise within the tumour during its development; hence, a similar approach for subtyping of bladder tumours might be useful. However, further studies are required to evaluate whether continuous scores might provide a more clinically relevant classification of bladder tumours than the current subtype likelihood scores.

The rapid developments in single-cell sequencing approaches might enable better delineations and more granular definitions of subtypes than those from bulk tumour analyses. No large studies of single cell RNA sequencing (RNA-seq) have been published for bladder cancer, but, in colorectal cancer, specific T cells, identified by single-cell

analysis, were found to be preferentially enriched in patients with microsatellite-instable tumours, which might explain favourable responses to immune checkpoint blockade in patients with these tumours[70]. In melanoma, single-cell RNA-seq revealed a resistance programme in malignant cells that is associated with T cell exclusion and immune evasion[71]. Specific cell subpopulations will probably not be identified from bulk tumour analysis and the high-resolution cellular maps of tumours generated from single-cell analysis might be crucial for an improved understanding of gene expression heterogeneity and subtype differences and for identification of better predictive biomarkers.

Morphology of heterogeneity

The genomic and transcriptional drivers that cause intratumoural heterogeneity are ultimately reflected in the varied histological morphologies of urothelial carcinoma. This heterogeneity can be observed in tumours that include more than one histological appearance within the same tumour. These morphologies reveal the wide spectrum of morphological heterogeneity in urothelial cancer and ultimately affect how a tumour responds to treatment[72–75]. Variants of urothelial carcinoma are divided on the basis of their microscopic morphological features, but increasing knowledge of the genetic and transcriptional attributes of histological variants have led to a better understanding of the molecular features associated with a subset of these lesions. The morphological spectrum of urothelial carcinoma includes divergent differentiation, such as squamous and glandular, as well as variant histologies such as nested, plasmacytoid, micropapillary, sarcomatoid and small-cell (neuroendocrine) carcinoma. Rarely, tumours of non-urothelial histology can develop in the bladder, such as squamous cell carcinoma and adenocarcinoma (FIG. 3).

Urothelial carcinoma with divergent differentiation.

The two most common variants of divergent differentiation in urothelial carcinoma are squamous and/or glandular differentiation[76]. Squamous differentiation is the most common variant, occurring in up to 30% of high-grade and/or high-stage urothelial carcinomas. In urothelial carcinoma with squamous differentiation, expression profile analysis of separate areas of urothelial and squamous morphology in the same tumour classified urothelial areas as luminal and squamous areas as basal/squamous in a subset of cases[4,77]. An association of squamous differentiation with human papillomavirus infection has been explored, but little genomic information exists to support a viral origin[72,75]. However, rarely, such as in patients with neurogenic bladder dysfunction or those requiring repeated catheterization, a viral aetiology has been identified, supported by p16 expression and human papillomavirus in situ hybridization[78,79].

The presence of glandular differentiation in urothelial carcinoma is less common than squamous differentiation (8–18% of high-grade tumours)[80]. Morphologically, the glandular component resembles adenocarcinomas of other organs, most commonly showing features of enteric or colonic adenocarcinoma, but can also rarely resemble mucinous or various mixed types of glandular morphology. One analysis of the molecular features of glandular differentiation in urothelial carcinoma has revealed high rates of hotspot mutations in the *TERT* promoter region, similar to urothelial carcinoma ithout glandular differentiation[81].

Nested urothelial carcinoma.

This rare variant of urothelial carcinoma belongs to a group of morphologically deceptively bland tumours that can be associated with an aggressive clinical course[82]. The morphological characteristics of nested urothelial carcinoma include the presence of invasive clusters of tumour cells without considerable morphological atypia or generally not associated with a stromal reaction. Identifying this variant can be challenging, as it is shares features with benign conditions, such as proliferative cystitis, von Brunn nest hyperplasia, nephrogenic adenoma or inverted papilloma[83,84]. To date, a high rate of *TERT* promoter mutations, which was not found in benign mimickers, was the only molecular finding in this tumour type[85], suggesting that it has molecular alterations similar to those of urothelial carcinoma in general.

Plasmacytoid urothelial carcinoma.

The plasmacytoid variant of urothelial carcinoma is another rare but aggressive tumour primarily composed of discohesive, infiltrating cells that resemble plasma cells, mostly admixed with other cells that contain intracytoplasmic vacuoles, giving the appearance of signet ring cells[86–88]. Patients with this tumour typically present at an advanced stage and have low survival. This tumour is also associated with high relapse rates and frequent peritoneal carcinomatosis, although patients might initially respond to chemotherapy[86–90].

This tumour shares immunohistochemical and genetic features with urothelial carcinoma. It frequently expresses markers of urothelial differentiation, such as CK7, p63, GATA3 and uroplakins, and generally has genetic alterations similar to those of urothelial carcinoma, such as mutations in *TP53, RB1, KMT2D* and ARID1A[86–90]. However, in contrast to urothelial carcinoma, loss-of-function mutations in *CDH1,* and less commonly promoter hypermethylation of *CDH1,* drive the development of this variant and contribute to its aggressive biology[86]. Targeted next-generation sequencing of macrodissected areas of plasmacytoid and urothelial histologies from the same tumour revealed shared mutations, suggesting that both arose from the same origin, but *CDH1* mutation was limited to the plasmacytoid component, supporting the role of *CDH1* loss in the development of this variant histology[86].

Micropapillary urothelial carcinoma.

The designation of micropapillary carcinoma requires the presence of small tight tumour clusters without true fibrovascular cores located within clear spaces, which is the result of reverse cellular orientation or polarization[91–93] and a lack of cohesion between tumour and stroma. This tumour type is generally associated with high rates of *ERBB2* alterations, mostly amplification[94] and less commonly mutations[95]. Morphological intratumoural heterogeneity in micropapillary carcinoma is common, as most of these tumours also contain a component of not otherwise specified (NOS) urothelial carcinoma[72]. In addition, intratumoural heterogeneity of *ERBB2* amplification is common. In tumours with mixed micropapillary and NOS urothelial carcinoma, *ERBB2* amplification was more common in micropapillary than NOS urothelial carcinoma components[96]. Additionally, the rate of *ERBB2* amplification in the NOS urothelial carcinoma component associated with

micropapillary components was much higher than that in NOS urothelial carcinoma not mixed with micropapillary components[40,97,98].

Sarcomatoid urothelial carcinoma.

When a component of urothelial carcinoma takes the form of a mesenchymal neoplasm, the tumour is designated sarcomatoid urothelial carcinoma, which is a rare form of bladder cancer that is typically associated with an advanced stage and overall poor prognosis[99]. The presence of a sarcomatous component in urothelial carcinoma does not exclude other variant histologies, as tumours with urothelial, glandular, squamous and/or small-cell or neuroendocrine differentiation have been reported[72]. The most common morphology of the sarcomatous component is that of spindle-cell proliferation, but it can also include myxoid, pseudoangiosarcomatous, undifferentiated pleomorphic sarcoma-like morphology, and true heterologous elements in the form of cartilaginous, osseous and other elements[100]. The sarcomatous and urothelial components within the same tumour have been reported to share a common clonal origin[101]. A comprehensive study of sarcomatoid urothelial carcinoma showed that this tumour type is enriched with mutations in *TP53, RB1* and *PIK3CA* and is associated with dysregulation of the epithelial-mesenchymal transition pathway and overexpression of epithelial-mesenchymal transition markers[100,102].

Small-cell (neuroendocrine) carcinoma.

Small-cell carcinoma is a rare form of urothelial carcinoma and can be admixed with a urothelial (invasive or non-invasive), glandular, squamous of sarcomatous component[72]. Its morphological appearance is similar to small-cell carcinomas of other organs; similarly, it commonly harbours co-alterations in both *TP53* and *RB1* (REFS[97,103,104]). However, *TP53* and *RB1* mutations are insufficient to explain development of small-cell carcinomas of the bladder, as these genetic alterations also often occur in urothelial carcinoma that does not exhibit features of small-cell or neuroendocrine differentiation. Furthermore, other alterations detected in urothelial carcinoma are also found in small-cell carcinoma of the bladder, in contrast to small-cell carcinoma of other organs. These aberrations include *TERT* promoter mutations in ~95% of samples and truncating alterations within chromatin remodelling genes, such as *CREBBP, EP300, ARID1A* and *KMT2D*, in nearly 75% of samples[104]. A high level of chromosomal instability is observed in bladder small-cell carcinoma, including whole genome duplication in 72% of tumours. Similar to MIBC, the APOBEC mutation signature is present in 95% of small-cell carcinoma of the bladder[20], which contrasts with small-cell carcinoma of the lung, whose mutation signature is typically associated with tobacco exposure[104]. These findings suggest that bladder small-cell carcinoma develops through transdifferentiation from urothelial carcinoma, but the exact molecular mechanisms for this transition are not yet clear[20,97,105]. In contrast to, for example, prostate cancer, where neuroendocrine differentiation almost always develops in the setting of androgen receptor-directed therapy[106], neuroendocrine differentiation in the bladder seems to develop de novo. Studies based on RNA and immunohistochemical expression profiling have identified a subtype of bladder cancer that is enriched in neuroendocrine markers but does not have the microscopic appearance of a true small-cell or neuroendocrine carcinoma[107,108]. This subtype has been referred to as “neuronal subtype” by TCGA classification[40] and “neuroendocrine-like” by a consensus clustering

recommendation of the Bladder Cancer Molecular Taxonomy Group[57]. Whether this subtype represents an early step in the development of frank neuroendocrine carcinoma is yet to be determined.

Adenocarcinoma.

The presence of pure glandular morphology is required for the diagnosis of primary adenocarcinoma of the bladder, which can develop anywhere in the bladder. If such a tumour develops in the bladder dome and is associated with a urachal remnant, it will be designated as urachal adenocarcinoma[109]. Most of these tumours resemble colorectal adenocarcinomas, but they can also resemble adenocarcinoma of any other organs. Genetically, adenocarcinomas are different from urothelial carcinoma, as they generally lack mutations in chromatin-modifying genes and the *TERT* promoter region and resemble a subset of colorectal adenocarcinoma that is enriched in mutations in *TP53, KRAS* and *SMAD4*, as well as a small subset with *EGFR* and *ERBB2* amplification[110–112].

Heterogeneity, systemic therapy and drug design

Tumour heterogeneity complicates systemic therapy, drug development and delivery owing to the potential variable response to therapeutics in different tumour regions[8,113]. Relapse is often associated with the emergence of resistance and resistance can occur because of intratumoural heterogeneity, and not only because of drug resistance mechanisms or the presence of natively resistant populations, such as cancer stem cells[114,115]. Subtyping of muscle-invasive urothelial carcinoma on the basis of gene expression profiles provides an opportunity for personalized medicine[4,55,58]. Similarly, expression profiles of NMIBC might enable subclassification of these tumours, but the therapeutic implications have not yet been explored[46]. Histological variants of MIBC, such as the micropapillary variant, might have distinct genetic profiles (for example, *ERBB2* overexpression), but whether these correlate with response to certain therapeutics (for example, HER2-targeted agents) has not been determined[116]. Defining which subtypes represent well-delineated groups, either natively or after therapy, remains a priority in the field.

Neoadjuvant chemotherapy and chemoradiotherapy.

Cisplatin-based regimens that comprise multiple agents are the most effective chemotherapy in advanced urothelial carcinoma[117]. Following evidence of improved survival of patients with metastatic bladder cancer treated with cisplatin chemotherapy, prospective randomized trials have demonstrated improved survival of patients with MIBC treated with neoadjuvant cisplatin-based chemotherapy[118,119]. Neoadjuvant chemotherapy is the current standard of care, but positive responses to immune checkpoint inhibitors (ICIs) in patients with MIBC and metastatic urothelial carcinoma have altered the neoadjuvant landscape to include immunotherapy[120]. Thus, determining the response of each tumour subtype to neoadjuvant chemotherapy and/or immunotherapy will be critical in determining the most efficacious precision therapy. For example, patients with basal-subtype tumours have the most improved survival benefit from neoadjuvant chemotherapy (3-year overall survival without neoadjuvant chemotherapy 49.2% versus 77.8% with neoadjuvant chemotherapy)[59], whereas those with luminal tumours treated without systemic therapy have the lowest rate of

upstaging compared with those with nonluminal tumours (34% versus 51%)[121]. Subtype assignment is usually considered absolute, but some subtypes are more ‘stable’ than others, meaning that repeat bio-informatic analysis of tumours with these designated subtypes is likely to result in the same subtype assignment. By contrast, subtypes such as luminal infiltrated tumours contain varying amounts of stroma and immune cells and are ‘unstable, with an increased likelihood of being designated as other subtypes in repeat clustering[4,68]. Thus, complexity at the cellular level is likely to affect subtype membership, which might have the greatest influence on the choice of neoadjuvant systemic therapy.

Targeted therapy.

Relatively few MIBCs are driven by single-gene drivers, with the exception of *FGFR3, RAS* and *PPARG*, which are predominantly found in luminal-subtype tumours[40]. BLC2001, a phase II dose-escalation study of the FGFR3-targeted agent erdafitinib in 99 patients, demonstrated an overall response rate of 34% and a median duration of response of 5.5 months in patients with metastatic urothelial cancer that harbours FGFR3 mutations and overexpression[122,123]. On the basis of these results, erdafitinib was approved by the FDA in 2019 for the treatment of patients with locally advanced or metastatic bladder cancer with *FGFR3* or *FGFR2* alterations and has progressed following platinum-containing chemotherapy[122]. In addition, in a phase II trial, the pan-FGFR inhibitor infigratinib demonstrated a 25.4% response rate and a 38.8% disease stabilization rate in patients with metastatic urothelial carcinoma and FGFR alterations[31]. Response to small molecular therapies targeting mutations or activation of FGFR might depend on intratumoural heterogeneity. For example, an evaluation of 27 MIBCs found FGFR3 alterations in ~30%, but only ~15% had FGFR3 alterations at deep tissue levels[124]. In addition, activating mutations of *PPARG* are common in MIBC (up to 15% of patients)[125]. Targeting of PPARG in urothelial carcinoma cell lines showed that inverse agonism of PPARG reduced proliferation rates of *PPARG*-mutant cells but not *PPARG* wild-type cells[125], pointing to another strategy by which patients with luminal tumours might benefit from targeted therapy in addition to established benefit from chemotherapy[125].

Response to immunotherapy.

Immunotherapy is contingent upon T cell infiltration in response to neoantigen expression[61,126]. Neoadjuvant immunotherapy in melanoma indicates that ICIs alter intratumoural heterogeneity, in part through selection of specific populations with a low antigen load, and might be more efficacious in regions with high immunogenicity[127]. In the phase II trial PURE-01 in patients with MIBC, treatment with the ICI pembrolizumab before cystectomy resulted in a reduction in total mutation burden or neoantigens in matched tumour samples, indicating removal of tumours with high mutational burden[128]. Thus, one conceivable mechanism of developed resistance to ICIs is a change in the neoantigen burden and the type of alterations found across a tumour after ICI treatment.

Clinical trial considerations

As our understanding of the molecular features that differentiate types of bladder cancer improves, biomarkers are likely to have an expanded role in future clinical trials. To improve

their accuracy, the reliability of both prognostic and predictive biomarkers in the setting of tumour heterogeneity is being investigated. Clinical decisions are made on the presence or absence of a biomarker and the accuracy of this biomarker to represent a treatment response of the tumour is critically dependent on the heterogeneity of the tested sample. One contemporary example in bladder cancer is the SWOG S1314 (co-expression extrapolation) study[129]. This randomized phase II trial includes 167 evaluable patients with non-metastatic MIBC and uses an Affymetrix gene expression model. Two separate models were tested (one for gemcitabine plus cisplatin (GC) and one for a dose-dense combination of methotrexate, vinblastine, doxorubicin and cisplatin (ddMVAC)) as prognostic and predictive biomarkers in these patients randomized to GC or ddMVAC chemotherapy regimens. Early data suggest that the individual GC and ddMVAC biomarkers do not predict response in their individual treatment arms but that the GC biomarker predicts response when applied to the larger, combined cohort of all patients (HR 2.33, 95% CI 1.11–4.89; $P = 0.02$)[130]. Future studies are essential to validate these results.

The S1314 study identified the challenges associated with heterogeneity that need to be addressed to optimize future biomarker-oriented bladder cancer clinical trials in the neoadjuvant setting. First, adequate tumour sampling, including depth of biopsy and multiple regions, will be required to evaluate the biomarker across multiple tumour sites. Depending on the tumour stage, a minimum tumour size or specifying the amount of viable tumour might be necessary. This might be easier for MIBC than for NMIBC, owing to the tumour burden, whereas the challenge in metastatic disease will be obtaining adequate tissue from needle biopsies. In multicentre trials, engaging local pathologists might also be advantageous, for example, to enable separate verification of an adequate amount of viable tissue as part of determining eligibility for inclusion in the trial. Second, the use of archival tissues versus the requirement for new tissue biopsy is an important consideration. Biologically, current assessment is desirable, as it minimizes aspects of temporal heterogeneity. However, in practice, requiring a new tissue biopsy of a metastatic site might put patients at increased risk and potential discomfort, making the patient less likely to participate in the trial.

The use of blood-based biopsies in clinical trials is quickly emerging as a new option[131]. This approach has the potential to obviate many concerns around the assessment of temporal heterogeneity, as contemporary sampling is easier than with tissue biopsies. In addition, the utility of urine analysis for tumour DNA continues to be investigated. This approach might enable direct measurement of tumour burden and identify specific dominant clones. For example, in patients with NMIBC, serial evaluation of urine specimens might identify drivers of persistence and recurrence during intravesical therapy. The utility of this strategy depends on the identification of cancer drivers and heterogeneity in NMIBC. For example, the currently limited genomic evaluation of Ta and T1 tumours suggests that *FGFR3* alterations are a dominant driver, but the clonality of this mutation during selective intravesical treatment remains unclear[132]. However, the handling of urine specimens requires special methods to avoid lysis of white blood cells and preferentially sampling urine, for example, in the morning to avoid increased dilution of cell-free DNA. Furthermore, identification of novel actionable targets through liquid biopsy analysis has been demonstrated in patients with metastatic bladder cancer and could complement current

patient selection procedures for clinical trials. A study in 68 patients with MIBC demonstrated an association between circulating tumour DNA fluctuations and chemotherapy response, which might enable more efficient treatment response evaluations in clinical trials than the current standard of radiographical imaging[133]. Thus, blood-based evaluation of circulating DNA might be a dynamic method of capturing a snapshot of tumour heterogeneity and inform changes in treatment owing to tumour evolution.

Conclusions

Our current understanding of the development and progression of urothelial carcinoma at the genetic and molecular levels indicates that single-agent therapy is unlikely to be successful, predominantly owing to intratumoural and intertumoural heterogeneity. This heterogeneity occurs at multiple levels and is best demonstrated in tumours with variant histology. Therapeutic targeting of the primary and metastatic lesions might require analysis of solid tumour or liquid biopsy samples to identify the evolving landscape of clones. Metastatic cells might have different subtypes and therapy might need to be further tailored as we begin to understand these populations. New therapeutics are still needed as response rates to agents currently in clinical trials remain suboptimal. As new drug candidates are being developed, designing trials that include those patients who are most likely to benefit and developing biomarkers to define these patients will be imperative.

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References

1. McGranahan N & Swanton C Clonal heterogeneity and tumor evolution: past, present, and the future. Cell 168, 613–628 (2017). [PubMed: 28187284]
2. Lamy P et al. Paired exome analysis reveals clonal evolution and potential therapeutic targets in urothelial carcinoma. Cancer Res. 76, 5894–5906 (2016). [PubMed: 27488526]
3. da Costa JB et al. Molecular tumor heterogeneity in muscle invasive bladder cancer: biomarkers, subtypes, and implications for therapy. Urol. Oncol 10.1016/j.urolonc.2018.11.015 (2018).
4. Warrick JI et al. Intratumoral heterogeneity of bladder cancer by molecular subtypes and histologic variants. Eur. Urol 75, 18–22 (2019). [PubMed: 30266310]
5. Ma G et al. Precision medicine and bladder cancer heterogeneity. Bull. Cancer 105, 925–931 (2018). [PubMed: 30243478]
6. Liu D et al. Mutational patterns in chemotherapy resistant muscle-invasive bladder cancer. Nat. Commun 8, 2193 (2017). [PubMed: 29259186]
7. Jamal-Hanjani M et al. Tracking the evolution of non-small-cell lung cancer. N. Engl. J. Med 376, 2109–2121 (2017). [PubMed: 28445112]
8. Gerlinger M et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N. Engl. J. Med 366, 883–892 (2012). [PubMed: 22397650]
9. Russo M et al. Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer. CancerDiscov. 6, 147–153 (2016).

10. Marks E, Rizvi SM, Sarwani N, Yang Z & El-Deiry WS A case of heterogeneous sensitivity to panitumumab in cetuximab-refractory colorectal adenocarcinoma metastases. Cancer Biol. Ther 16, 377–382 (2015). [PubMed: 25695537]
11. Pauli C et al. Personalized in vitro and in vivo cancer models to guide precision medicine. Cancer Discov. 7, 462–477 (2017). [PubMed: 28331002]
12. Babjuk M et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2016. Eur. Urol 71, 447–461 (2017). [PubMed: 27324428]
13. Dyrskjøt L & Ingersoll MA Biology of nonmuscle-invasive bladder cancer: pathology, genomic implications, and immunology. Curr. Opin. Urol 28, 598–603 (2018). [PubMed: 30138123]
14. Hurst CD & Knowles MA Mutational landscape of non-muscle-invasive bladder cancer. Urol. Oncol 10.1016/j.urolonc.2018.10.015 (2018).
15. Gerlinger M et al. Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. Nat. Genet 46, 225–233 (2014). [PubMed: 24487277]
16. Epstein JI et al. The 2014 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma: definition of grading patterns and proposal for a new grading system. Am. J. Surg. Pathol 40, 244–252 (2016). [PubMed: 26492179]
17. Faltas BM et al. Clonal evolution of chemotherapy-resistant urothelial carcinoma. Nat. Genet 48, 1490–1499 (2016). [PubMed: 27749842]
18. Yachida S et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 467, 1114–1117 (2010). [PubMed: 20981102]
19. McPherson A et al. Divergent modes of clonal spread and intraperitoneal mixing in high-grade serous ovarian cancer. Nat. Genet 48, 758–767 (2016). [PubMed: 27182968]
20. The Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 507, 315–322 (2014). [PubMed: 24476821]
21. Findlay JM et al. Differential clonal evolution in oesophageal cancers in response to neo-adjuvant chemotherapy. Nat. Commun 7, 11111 (2016). [PubMed: 27045317]
22. McQuerry JA, Chang JT, Bowtell DDL, Cohen A & Bild AH Mechanisms and clinical implications of tumor heterogeneity and convergence on recurrent phenotypes. J. Mol. Med 95, 1167–1178 (2017). [PubMed: 28871446]
23. Meeks JJ, Goldkorn A, Aparicio AM & McConkey DJ Development of a translational medicine protocol for an NCTN genitourinary clinical trial: critical steps, common pitfalls and a basic guide to translational clinical research. Urol. Oncol 37, 313–317 (2019). [PubMed: 30115512]
24. Sharma M, Gogia A, Deo SSV & Mathur S Role of rebiopsy in metastatic breast cancer at progression. Curr. Probl. Cancer 43, 438–442 (2019). [PubMed: 30559028]
25. Hench IB, Hench J & Tolnay M Liquid biopsy in clinical management of breast, lung, and colorectal cancer. Front. Med 5, 9 (2018).
26. Christensen E et al. Early detection of metastatic relapse and monitoring of therapeutic efficacy by ultra-deep sequencing of plasma cell-free DNA in patients with urothelial bladder carcinoma. J. Clin. Oncol 37, 1547–1557 (2019). [PubMed: 31059311]
27. Thierry AR et al. Clinical utility of circulating DNA analysis for rapid detection of actionable mutations to select metastatic colorectal patients for anti-EGFR treatment. Ann. Oncol 28, 2149–2159 (2017). [PubMed: 28911069]
28. Agarwal N et al. Characterization of metastatic urothelial carcinoma via comprehensive genomic profiling of circulating tumor DNA. Cancer 124, 2115–2124 (2018). [PubMed: 29517810]
29. Paweletz CP et al. Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients. Clin. Cancer Res 22, 915–922 (2016). [PubMed: 26459174]
30. Barata PC et al. Next-generation sequencing (NGS) of cell-free circulating tumor DNA and tumor tissue in patients with advanced urothelial cancer: a pilot assessment of concordance. Ann. Oncol 28, 2458–2463 (2017). [PubMed: 28945843]
31. Pal SK et al. Efficacy of BGJ398, a fibroblast growth factor receptor 1–3 Inhibitor, in patients with previously treated advanced urothelial carcinoma with FGFR3 alterations. CancerDiscov. 8, 812–821 (2018).

32. Oellerich M et al. Using circulating cell-free DNA to monitor personalized cancer therapy. Crit. Rev Clin. Lab. Sci 54, 205–218 (2017). [PubMed: 28393575]
33. Chalmers ZR et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med. 9, 34 (2017). [PubMed: 28420421]
34. Nordentoft I et al. Mutational context and diverse clonal development in early and late bladder cancer. Cell Rep. 7, 1649–1663 (2014). [PubMed: 24835989]
35. Cha EK et al. Branched evolution and intratumor heterogeneity of urothelial carcinoma of the bladder. J. Clin. Oncol 32, 293–293 (2014).
36. Prandi D et al. Unraveling the clonal hierarchy of somatic genomic aberrations. Genome Biol. 15, 439 (2014). [PubMed: 25160065]
37. Thomsen MBH et al. Spatial and temporal clonal evolution during development of metastatic urothelial carcinoma. Mol. Oncol 10, 1450–1460 (2016). [PubMed: 27582092]
38. Dentro SC, Wedge DC & Van Loo P Principles of reconstructing the subclonal architecture of cancers. Cold SpringHarb. Perspect. Med 10.1101/cshperspect.a026625 (2017).
39. Waclaw B et al. A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. Nature 525, 261–264 (2015). [PubMed: 26308893]
40. Robertson AG et al. Comprehensive molecular characterization of muscle-invasive bladder cancer. Cell 171, 540–556 (2018).
41. Moris A, Murray S & Cardinaud S AID and APOBECs span the gap between innate and adaptive immunity. Front. Microbiol 5, 534 (2014). [PubMed: 25352838]
42. Swanton C, McGranahan N, Starrett GJ & Harris RS APOBEC enzymes: mutagenic fuel for cancer evolution and heterogeneity. Cancer Discov. 5, 704–712 (2015). [PubMed: 26091828]
43. Salter JD, Bennett RP & Smith HC The APOBEC protein family: united by structure, divergent in function. Trends Biochem. Sci 41, 578–594 (2016). [PubMed: 27283515]
44. Roberts SA et al. An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. Nat. Genet 45, 970–976 (2013). [PubMed: 23852170]
45. Burns MB, Temiz NA & Harris RS Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat. Genet 45, 977–983 (2013). [PubMed: 23852168]
46. Hedegaard J et al. Comprehensive transcriptional analysis of early-stage urothelial carcinoma. Cancer Cell 30, 27–42 (2016). [PubMed: 27321955]
47. Blanes A, Rubio J, Sanchez-Carrillo JJ & Diaz-Cano SJ Coexistent intraurothelial carcinoma and muscle-invasive urothelial carcinoma of the bladder: clonality and somatic down-regulation of DNA mismatch repair. Hum. Pathol 40, 988–997 (2009). [PubMed: 19368958]
48. Kazanov MD et al. APOBEC-induced cancer mutations are uniquely enriched in early-replicating, gene-dense, and active chromatin regions. Cell Rep. 13, 1103–1109 (2015). [PubMed: 26527001]
49. Hoopes JI et al. APOBEC3A and APOBEC3B preferentially deaminate the lagging strand template during DNA replication. Cell Rep. 14, 1273–1282 (2016). [PubMed: 26832400]
50. Seplyarskiy VB et al. APOBEC-induced mutations in human cancers are strongly enriched on the lagging DNA strand during replication. Genome Res. 26, 174–182 (2016). [PubMed: 26755635]
51. Nowarski R & Kotler M APOBEC3 cytidine deaminases in double-strand DNA break repair and cancer promotion. Cancer Res. 73, 3494–3498 (2013). [PubMed: 23598277]
52. Okeoma CM, Lovsin N, Peterlin BM & Ross SR APOBEC3 inhibits mouse mammary tumour virus replication in vivo. Nature 445, 927–930 (2007). [PubMed: 17259974]
53. Smith HC, Bennett RP, Kizilyer A, McDougall WM & Prohaska KM Functions and regulation of the APOBEC family of proteins. Semin. Cell Dev. Biol 23, 258–268 (2012). [PubMed: 22001110]
54. Law EK et al. The DNA cytosine deaminase APOBEC3B promotes tamoxifen resistance in ER-positive breast cancer. Sci. Adv 2, e1601737 (2016). [PubMed: 27730215]
55. Sjödahl G et al. A molecular taxonomy for urothelial carcinoma. Clin. Cancer Res 18, 3377–3386 (2012). [PubMed: 22553347]
56. Choi W et al. Genetic alterations in the molecular subtypes of bladder cancer: illustration in the cancer genome atlas dataset. Eur. Urol 72, 354–365 (2017). [PubMed: 28365159]
57. Kamoun A et al. A consensus molecular classification of muscle-invasive bladder cancer. Eur. Urol 77, 420–433 (2020). [PubMed: 31563503]

58. Choi W et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 25, 152–165 (2014). [PubMed: 24525232]
59. Seiler R et al. Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. Eur Urol. 72, 544–554 (2017). [PubMed: 28390739]
60. Mariathasan S et al. TGFß attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. Nature 554, 544–548 (2018). [PubMed: 29443960]
61. Rosenberg JE et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. Lancet 387, 1909–1920 (2016). [PubMed: 26952546]
62. Sharma P et al. Nivolumab in metastatic urothelial carcinoma after platinum therapy (CheckMate 275): a multicentre, single-arm, phase 2 trial. Lancet Oncol. 18, 312–322 (2017). [PubMed: 28131785]
63. Greaves M & Maley CC Clonal evolution in cancer. Nature 481, 306–313 (2012). [PubMed: 22258609]
64. Rousseeuw PJ Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. J. Comput. Appl. Math 20, 53–65 (1987).
65. Dyrskjøt L et al. A molecular signature in superficial bladder carcinoma predicts clinical outcome. Clin. Cancer Res 11, 4029–4036 (2005). [PubMed: 15930337]
66. Dyrskjøt L et al. Prognostic impact of a 12-gene progression score in non-muscle-invasive bladder cancer: a prospective multicentre validation study. Eur Urol. 72, 461–469 (2017). [PubMed: 28583312]
67. Sjödahl G et al. Discordant molecular subtype classification in the basal-squamous subtype of bladder tumors and matched lymph-node metastases. Mod. Pathol 31, 1869–1881 (2018). [PubMed: 29967424]
68. Thomsen MBH et al. Comprehensive multiregional analysis of molecular heterogeneity in bladder cancer. Sci. Rep 7, 11702 (2017). [PubMed: 28916750]
69. Ma S et al. Continuity of transcriptomes among colorectal cancer subtypes based on meta-analysis. Genome Biol. 19, 142 (2018). [PubMed: 30253799]
70. Zhang L et al. Lineage tracking reveals dynamic relationships of T cells in colorectal cancer. Nature 564, 268–272 (2018). [PubMed: 30479382]
71. Jerby-Arnon L et al. A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade. Cell 175, 984–997.e24 (2018). [PubMed: 30388455]
72. Amin MB Histological variants of urothelial carcinoma: diagnostic, therapeutic and prognostic implications. Mod. Pathol 22, S96–S118 (2009). [PubMed: 19494856]
73. Linder BJ et al. The impact of histological reclassification during pathology re-review — evidence of a Will Rogers effect in bladder cancer? J. Urol 190, 1692–1696 (2013). [PubMed: 23707452]
74. Shah RB, Montgomery JS, Montie JE & Kunju LP Variant (divergent) histologic differentiation in urothelial carcinoma is under-recognized in community practice: impact of mandatory central pathology review at a large referral hospital. Urol. Oncol 31, 1650–1655 (2013). [PubMed: 22608543]
75. Moch H, Humphrey PA, Ulbright TM & Reuter VE WHO Classification of Tumours of the Urinary System and Male Genital Organs Vol. 4, 77–133 (International Agency for Research on Cancer, 2016).
76. Humphrey PA, Moch H, Cubilla AL, Ulbright TM & Reuter VE The 2016 WHO classification of tumours of the urinary system and male genital organs — part B: prostate and bladder tumours. Eur. Urol 70, 106–119 (2016). [PubMed: 26996659]
77. Hovelson DH et al. Targeted DNA and RNA sequencing of paired urothelial and squamous bladder cancers reveals discordant genomic and transcriptomic events and unique therapeutic implications. Eur. Urol 74, 741–753 (2018). [PubMed: 30033047]
78. Blochin EB, Park KJ, Tickoo SK, Reuter VE & Al-Ahmadie H Urothelial carcinoma with prominent squamous differentiation in the setting of neurogenic bladder: role of human papillomavirus infection. Mod. Pathol 25, 1534–1542 (2012). [PubMed: 22766788]

79. Chapman-Fredricks JR et al. High-risk human papillomavirus DNA detected in primary squamous cell carcinoma of urinary bladder. Arch. Pathol. Lab. Med 137, 1088–1093 (2013). [PubMed: 23899065]
80. Solomon JP et al. Challenges in the diagnosis of urothelial carcinoma variants: can emerging molecular data complement pathology review? Urology 102, 7–16 (2016). [PubMed: 27769917]
81. Vail E et al. Telomerase reverse transcriptase promoter mutations in glandular lesions of the urinary bladder. Ann. Diagn. Pathol 19, 301–305 (2015). [PubMed: 26239299]
82. Lopez-Beltran A et al. Variants and new entities of bladder cancer. Histopathology 74, 77–96 (2019). [PubMed: 30565299]
83. Dhall D, Al-Ahmadie H & Olgac S Nested variant of urothelial carcinoma. Arch. Pathol. Lab. Med 131, 1725–1727 (2007). [PubMed: 17979494]
84. Volmar KE, Chan TY, De Marzo AM & Epstein JI Florid von Brunn nests mimicking urothelial carcinoma: a morphologic and immunohistochemical comparison to the nested variant of urothelial carcinoma. Am. J. Surg. Pathol 27, 1243–1252 (2003). [PubMed: 12960809]
85. Zhong M et al. Distinguishing nested variants of urothelial carcinoma from benign mimickers by TERT promoter mutation. Am. J. Surg. Pathol 39, 127–131 (2015). [PubMed: 25118812]
86. Al-Ahmadie HA et al. Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer. Nat. Genet 48, 356–358 (2016). [PubMed: 26901067]
87. Keck B et al. The plasmacytoid carcinoma of the bladder-rare variant of aggressive urothelial carcinoma. Int. J. Cancer 129, 346–354 (2011). [PubMed: 20878954]
88. Nigwekar P et al. Plasmacytoid urothelial carcinoma: detailed analysis of morphology with clinicopathologic correlation in 17 cases. Am. J. Surg. Pathol 33, 417–424 (2009). [PubMed: 19011563]
89. Dayyani F et al. Plasmacytoid urothelial carcinoma, a chemosensitive cancer with poor prognosis, and peritoneal carcinomatosis. J. Urol 189, 1656–1661 (2013). [PubMed: 23159581]
90. Kaimakliotis HZ et al. Plasmacytoid bladder cancer: variant histology with aggressive behavior and a new mode of invasion along fascial planes. Urology 83, 1112–1116 (2014). [PubMed: 24582117]
91. Amin MB et al. Micropapillary variant of transitional cell carcinoma of the urinary bladder. Histologic pattern resembling ovarian papillary serous carcinoma. Am. J. Surg. Pathol 18, 1224–1232 (1994). [PubMed: 7977945]
92. Nassar H et al. Pathogenesis of invasive micropapillary carcinoma: role of MUC1 glycoprotein. Mod. Pathol 17, 1045–1050 (2004). [PubMed: 15154007]
93. Luna-More S, Gonzalez B, Acedo C, Rodrigo I & Luna C Invasive micropapillary carcinoma of the breast. A new special type of invasive mammary carcinoma. Pathol. Res. Pract 190, 668–674 (1994). [PubMed: 7808965]
94. Ching CB et al. HER2 gene amplification occurs frequently in the micropapillary variant of urothelial carcinoma: analysis by dual-color in situ hybridization. Mod. Pathol 24, 1111–1119 (2011). [PubMed: 21516078]
95. Ross JS et al. A high frequency of activating extracellular domain ERBB2 (HER2) mutation in micropapillary urothelial carcinoma. Clin. Cancer Res 20, 68–75 (2014). [PubMed: 24192927]
96. Isharwal S et al. Intratumoral heterogeneity of ERBB2 amplification and HER2 expression in micropapillary urothelial carcinoma. Hum. Pathol 77, 63–69 (2018). [PubMed: 29601842]
97. Iyer G et al. Prevalence and co-occurrence of actionable genomic alterations in high-grade bladder cancer. J. Clin. Oncol 31, 3133–3140 (2013). [PubMed: 23897969]
98. Fleischmann A, Rotzer D, Seiler R, Studer UE & Thalmann GN Her2 amplification is significantly more frequent in lymph node metastases from urothelial bladder cancer than in the primary tumours. Eur. Urol 60, 350–357 (2011). [PubMed: 21640482]
99. Lopez-Beltran A et al. Carcinosarcoma and sarcomatoid carcinoma of the bladder: clinicopathological study of 41 cases. J. Urol 159, 1497–1503 (1998). [PubMed: 9554341]
100. Sanfrancesco J et al. Sarcomatoid urothelial carcinoma of the bladder: analysis of 28 cases with emphasis on clinicopathologic features and markers of epithelial-to-mesenchymal transition. Arch. Pathol. Lab. Med 140, 543–551 (2016). [PubMed: 27031776]

101. Sung MT et al. Histogenesis of sarcomatoid urothelial carcinoma of the urinary bladder: evidence for a common clonal origin with divergent differentiation. J. Pathol 211, 420–430 (2007). [PubMed: 17236170]
102. Guo CC et al. Dysregulation of EMT drives the progression to clinically aggressive sarcomatoid bladder cancer. Cell Rep. 27, 1781–1793.e4 (2019). [PubMed: 31067463]
103. George J et al. Comprehensive genomic profiles of small cell lung cancer. Nature 524, 47–53 (2015). [PubMed: 26168399]
104. Chang MT et al. Small cell carcinomas of the bladder and lung are characterized by a convergent but distinct pathogenesis. Clin. Cancer Res 24, 1965–1973 (2018). [PubMed: 29180607]
105. Kim PH et al. Genomic predictors of survival in patients with high-grade urothelial carcinoma of the bladder. Eur. Urol 67, 198–201 (2015). [PubMed: 25092538]
106. Beltran H et al. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. Nat. Med 22, 298–305 (2016). [PubMed: 26855148]
107. Sjödahl G, Eriksson P, Liedberg F & Höglund M Molecular classification of urothelial carcinoma: global mRNA classification versus tumour-cell phenotype classification. J. Pathol 242, 113–125 (2017). [PubMed: 28195647]
108. Batista da Costa J et al. Molecular characterization of neuroendocrine-like bladder cancer. Clin. Cancer Res 25, 3908–3920 (2019). [PubMed: 30952638]
109. Gopalan A et al. Urachal carcinoma: a clinicopathologic analysis of 24 cases with outcome correlation. Am. J. Surg. Pathol 33, 659–668 (2009). [PubMed: 19252435]
110. Jordan E et al. Assessment of genomic alterations in bladder adenocarcinoma and urachal adenocarcinoma. Eur. J. Cancer 51, S530–S530 (2015).
111. Collazo-Lorduy A et al. Urachal carcinoma shares genomic alterations with colorectal carcinoma and may respond to epidermal growth factor inhibition. Eur. Urol 70, 771–775 (2016). [PubMed: 27178450]
112. Reis H et al. Pathogenic and targetable genetic alterations in 70 urachal adenocarcinomas. Int. J. Cancer 143, 1764–1773 (2018). [PubMed: 29672836]
113. Saunders NA et al. Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. EMBO Mol. Med 4, 675–684 (2012). [PubMed: 22733553]
114. Garraway LA & Janne PA Circumventing cancer drug resistance in the era of personalized medicine. CancerDiscov. 2, 214–226 (2012).
115. Steinbichler TB et al. Therapy resistance mediated by cancer stem cells. Semin. Cancer Biol 53, 156–167 (2018). [PubMed: 30471331]
116. Guo CC et al. Gene expression profile of the clinically aggressive micropapillary variant of bladder cancer. Eur. Urol 70, 611–620 (2016). [PubMed: 26988609]
117. Meeks JJ et al. A systematic review of neoadjuvant and adjuvant chemotherapy for muscle-invasive bladder cancer. Eur. Urol 62, 523–533 (2012). [PubMed: 22677572]
118. Grossman HB et al. Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. N. Engl. J. Med 349, 859–866 (2003). [PubMed: 12944571]
119. Plimack ER et al. Accelerated methotrexate, vinblastine, doxorubicin, and cisplatin is safe, effective, and efficient neoadjuvant treatment for muscle-invasive bladder cancer: results of a multicenter phase II study with molecular correlates of response and toxicity. J. Clin. Oncol 32, 1895–1901 (2014). [PubMed: 24821881]
120. Kamat AM et al. Bladder cancer. Lancet 388, 2796–2810 (2016). [PubMed: 27345655]
121. Lotan Y et al. Molecular subtyping of clinically localized urothelial carcinoma reveals lower rates of pathological upstaging at radical cystectomy among luminal tumors. Eur. Urol 76, 200–206 (2019). [PubMed: 31092337]
122. The ASCO Post. FDA grants accelerated approval to erdafitinib for metastatic urothelial carcinoma. The ASCO Post https://www.ascopost.com/News/59934 (2019).
123. Loriot Y et al. Erdafitinib in locally advanced or metastatic urothelial carcinoma. N. Engl. J. Med 381, 338–348 (2019). [PubMed: 31340094]

124. Pouessel D et al. Tumor heterogeneity of fibroblast growth factor receptor 3 (FGFR3) mutations in invasive bladder cancer: implications for perioperative anti-FGFR3 treatment. Ann. Oncol 27, 1311–1316 (2016). [PubMed: 27091807]

125. Goldstein JT et al. Genomic activation of PPARG reveals a candidate therapeutic axis in bladder cancer. Cancer Res. 77, 6987–6998 (2017). [PubMed: 28923856]

126. Siefker-Radtke A & Curti B Immunotherapy in metastatic urothelial carcinoma: focus on immune checkpoint inhibition. Nat. Rev. Urol 15, 112–124 (2018). [PubMed: 29205200]

127. Riaz N et al. Tumor and microenvironment evolution during immunotherapy with nivolumab. Cell 171, 934–949.e16 (2017). [PubMed: 29033130]

128. Necchi A et al. Pembrolizumab as neoadjuvant therapy before radical cystectomy in patients with muscle-invasive urothelial bladder carcinoma (PURE-01): an open-label, single-arm, phase II study. J. Clin. Oncol 6, 3353–3360 (2018).

129. US National Library of Medicine. ClinicalTrials.gov http://www.clinicaltrials.gov/ct2/show/NCT02177695 (2019).

130. Flaig TW et al. SWOG S1314: a randomized phase II study of co-expression extrapolation (COXEN) with neoadjuvant chemotherapy for localized, muscle-invasive bladder cancer. J. Clin. Oncol 37, 4506–4506 (2019).

131. Christensen E et al. Early detection of metastatic relapse and monitoring of therapeutic efficacy by ultra-deep sequencing of plasma cell-free DNA in patients with urothelial bladder carcinoma. J. Clin. Oncol 37, 1547–1557 (2019). [PubMed: 31059311]

132. Hurst CD et al. Genomic subtypes of non-invasive bladder cancer with distinct metabolic profile and female gender bias in KDM6A mutation frequency. Cancer Cell 32, 701–715.e7 (2017). [PubMed: 29136510]

133. Vandekerkhove G et al. Circulating tumor DNA reveals clinically actionable somatic genome of metastatic bladder cancer. Clin. Cancer Res 23, 6487–6497 (2017). [PubMed: 28760909]

Key points

- Bladder cancers have a high total mutational burden and considerable intratumoural and intertumoural heterogeneity at the genomic, transcriptional and cellular levels that remain difficult to quantify.
- Heterogeneity might be driven by genomic events initiated by APoBEC enzymes and selection pressure from therapeutic interventions, which both drive tumour evolution.
- Bladder tumours can be categorized into different subtypes on the basis of gene expression signatures, but these molecular subtypes might be unstable and different subtypes can occur within the same tumour causing intratumoural heterogeneity.
- variant tumour histologies are the morphological extreme of tumour heterogeneity and include glandular, squamous, nested, plasmacytoid, micropapillary, sarcomatoid and small-cell carcinoma.
- Tumour heterogeneity might affect treatment efficacy, for example, of neoadjuvant chemotherapy and immune checkpoint inhibitors, as well as targeted therapy, for example, when individual actionable mutations only occur in a fraction of the tumour.
- Biomarkers to select personalized treatments in precision medicine approaches will likely shape future clinical trial design, but their validity might be affected by heterogeneity.

Box 1 |

Definitions of heterogeneity

- Heterogeneity: the state of comprising diverse or dissimilar elements
- Interpatient heterogeneity: differences between tumours of patients diagnosed with the same type of primary cancer
- Intratumoural heterogeneity: differences between spatial regions of the primary tumour of the same patient
- Intertumoural heterogeneity: differences between multiple primary tumours in the same patient, the primary tumour and metastatic deposits or multiple metastatic sites
- Temporal heterogeneity: molecular or genetic changes in the tumour over time and/or during treatment of the same patient
- Circulation heterogeneity: differences between circulating tumour markers and tissue-based tumour markers

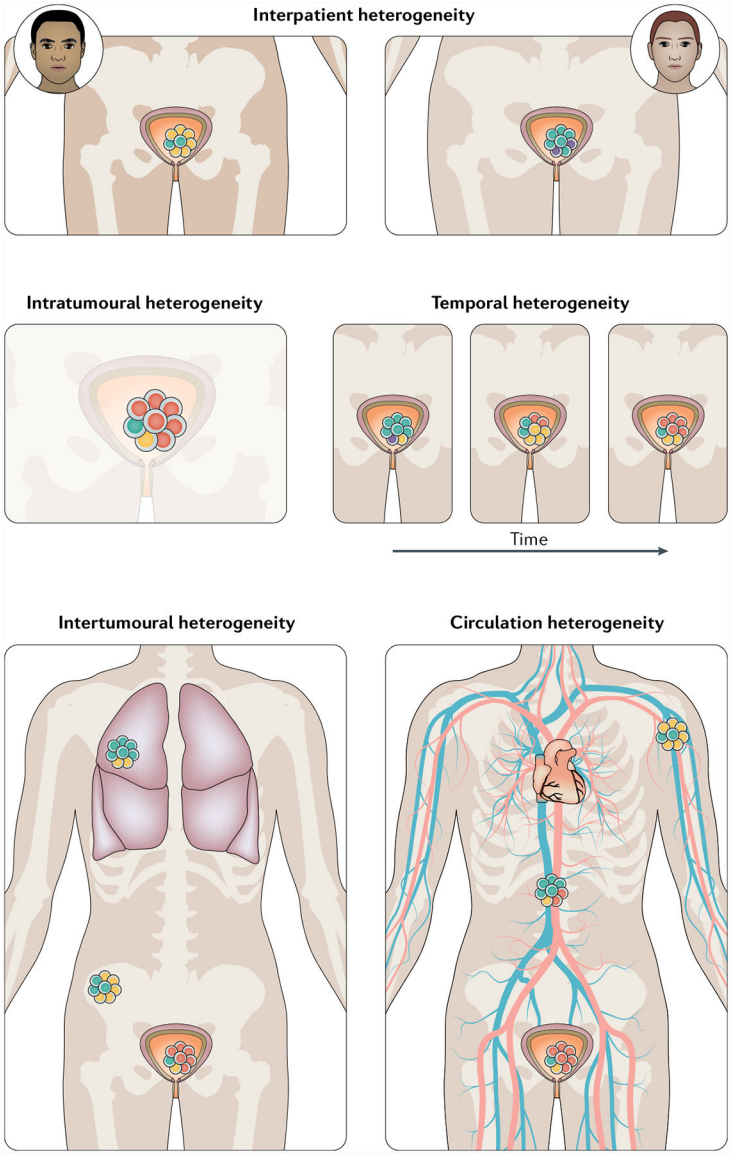

Fig. 1 |. Different types of heterogeneity found in bladder cancer.

Bladder tumours can vary in morphology, gene expression profile and mutations. This heterogeneity exists not only between patients (interpatient heterogeneity) but also within the same patient, where subclassifications can be made. Intratumoural heterogeneity describes variations between regions of one tumour and can be affected or caused by clonality, immune cell infiltration and the tumour microenvironment. Differences between multiple tumours and/or metastases within one patient are termed intertumoural heterogeneity. Heterogeneity can also change over time and during treatment (temporal heterogeneity). Finally, differences can also exist between tissue-based tumour markers and circulating markers (circulation heterogeneity) and can be assessed by comparing data from tumour deposits with those from liquid biopsy approaches.

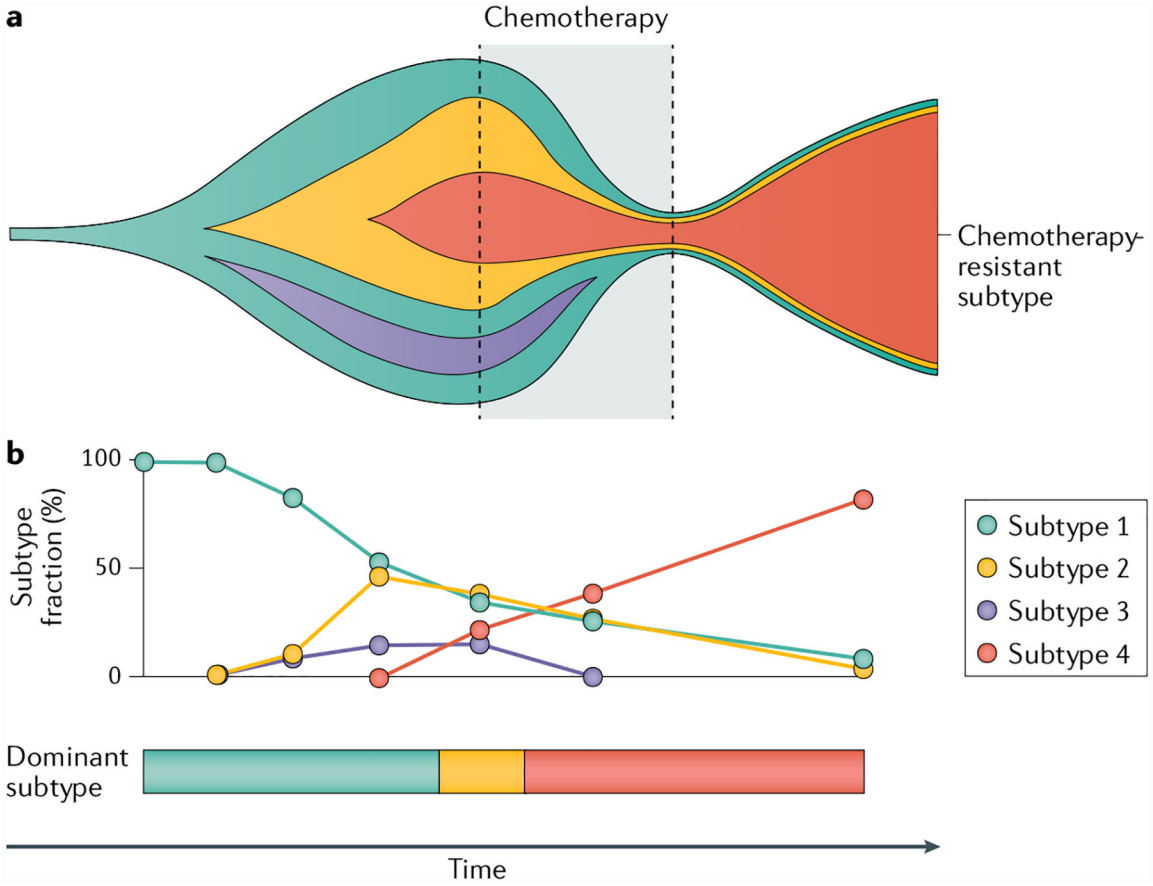

Fig. 2 |. Tumour evolution with emergence of distinct tumour subtypes.

a | Schematic of the occurrence of tumour subclones over time. Genomic differences between subclones might result in different expression patterns and thereby different tumour subtypes. Chemotherapy can cause contraction of subtypes and isolation of a specific subtype, which becomes the dominant clone after chemotherapy (subtype 4 in this example). **b** | The fractions of different subtypes can vary over time and under the selection pressure of treatments, resulting in inconsistent subtype calling even if the entire tumour is analysed. Sampling of only parts of a tumour would be expected to further complicate consistent subtype calling.

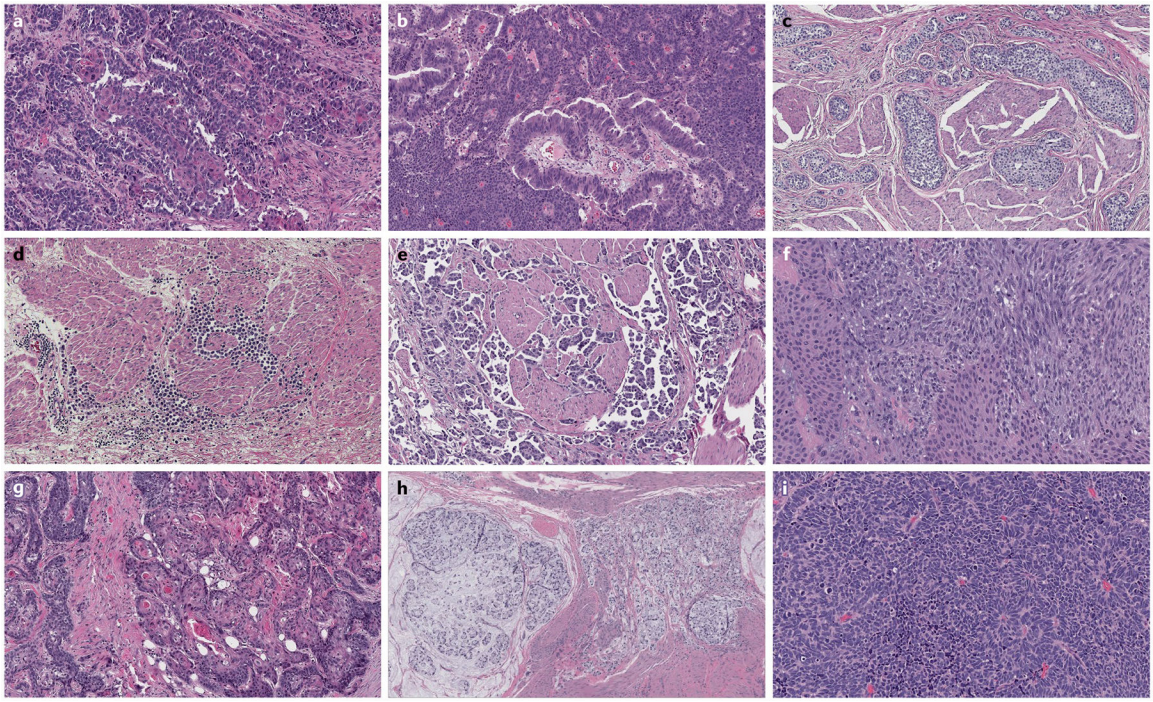

Fig. 3 |. Variant histology of urothelial carcinoma.

Tumour heterogeneity is most pronounced at the morphological level when comparing urothelial carcinomas with variant histology. The morphological spectrum of urothelial carcinoma includes divergent differentiation, such as squamous differentiation (part **a**) and glandular differentiation (part **b**). Variant histologies of urothelial carcinoma include nested variant (part **c**), plasmacytoid variant (part **d**), micropapillary variant (part **e**) and sarcomatoid variant (part **f**). Primary tumours of non-urothelial histology can also develop in the bladder, including squamous cell carcinoma (part **g**), mucinous adenocarcinoma with signet ring cells (part **h**) and small-cell or neuroendocrine carcinoma (part **i**). Haematoxylin and eosin staining in all images, magnification ×50.