Genomic and phenotypic heterogeneity in prostate cancer

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Abstract

From a clinical, morphological and molecular perspective, prostate cancer is a heterogeneous disease. Primary prostate cancers are often multifocal, having topographically and morphologically distinct tumour foci. Sequencing studies have revealed that individual tumour foci can arise as clonally distinct lesions with no shared driver gene alterations. This finding demonstrates that multiple genomically and phenotypically distinct primary prostate cancers can be present in an individual patient. Lethal metastatic prostate cancer seems to arise from a single clone in the primary tumour but can exhibit subclonal heterogeneity at the genomic, epigenetic and phenotypic levels. Collectively, this complex heterogeneous constellation of molecular alterations poses obstacles for the diagnosis and treatment of prostate cancer. However, advances in our understanding of intra-tumoural heterogeneity and the development of novel technologies will allow us to navigate these challenges, refine approaches for translational research and ultimately improve patient care.

Cancer cells show differences in many measurable traits, including proliferation, metastatic potential and therapeutic resistance. Such differences are determined by heritable genetic and epigenetic alterations[1,2]. Every patient's cancer has unique genomic and phenotypic changes, but over the past few years an increasing number of studies have highlighted the

high level of genetic and phenotypic heterogeneity within a given tumour[2–4]. These insights have been driven by advances in sequencing technology and molecular pathology approaches that have enabled the study of genomic and molecular alterations at an unprecedented resolution[5–7]. Applying methods adapted from evolutionary biology to such sequencing data has demonstrated the complex relationship of different lesions in primary tumours and metastases and provided evidence for independent clonal evolution of tumour cell subpopulations[8,9].

Prostate cancer is the most common non-skin malignancy diagnosed in men in the Western world and has long been known to be a heterogeneous disease[10]. The clinical presentation of prostate cancer can range from localized indolent to a rapidly progressing lethal metastatic disease[11–13]. Although the majority of men are diagnosed with organ-confined disease, long-term oncological outcomes can vary greatly[14–16]. Furthermore, histomorphological and molecular tumour characteristics show substantial diversity between different patients and within a given tumour. This poses diagnostic challenges and has major implications for clinical management[4,17,18].

Much of the complexity of primary prostate cancer diagnosis is rooted in the multifocal nature of the disease. Numerous reports have documented that >80% of primary prostate cancers show multiple topographically and histomorphologically distinct tumour foci[19–22]. Sequencing efforts have demonstrated a high level of genomic diversity between different patients (inter-patient heterogeneity) but also within a given primary tumour (intra-tumoural heterogeneity) as well as its distinct tumour foci and different metastatic sites (inter-tumoural heterogeneity)[4,23–27]. In addition to the complex presentation of primary tumours, metastatic disease shows a similar level of genomic and phenotypic heterogeneity[28–31], which complicates the assessment of molecular alterations and poses potential barriers to the implementation of precision medicine[32].

In this Review, we summarize the manifestations of inter-tumoural and intra-tumoural heterogeneity in primary and metastatic prostate cancer. We emphasize the significant contribution of genomics studies in this field and discuss the importance of phenotypic changes. Finally, we provide a framework for assessing heterogeneity and critically discuss the implications for clinical management and research.

Understanding heterogeneity

Within a given tumour, cancer cells and the tumour-associated microenvironment can show alterations in molecular characteristics[24]. All of these changes show a variable degree of difference between individual tumour cells, cell clusters or topographically or anatomically separated tumour lesions. Such heterogeneities can result in distinct cellular phenotypes that contribute to the overall biological behaviour of a tumour. Owing to advancements in DNA sequencing technologies over the past two decades, heterogeneity at the genetic level has been the focus of a large number of studies, which have revealed important insights into the genomic composition of many solid tumours (including prostate cancer) and allowed a delineation of clonal and subclonal relationships of different tumour cell populations[7,25,29,33,34]. These studies have shed light on the complex evolution and

competition of genomically defined tumour cell subpopulations during tumour progression and in response to therapy[2,7,8,25,29,34–36]. However, the term heterogeneity can be more broadly defined and applied to other measurable differences, not just at the genomic level[2,24]. Epigenetic, expression, post-translational, morphological and phenotypic heterogeneities, which all probably contribute to disease progression and clinical manifestations, exist within solid tumours. These non-genomic changes can be used to define heterogeneity, but they only allow an indirect assessment of the underlying clonal and subclonal changes.

Clonal and subclonal alterations.

All human life starts from a single cell that subsequently undergoes clonal expansions, ultimately giving rise to all organ systems. Therefore, all cells in the human body are monoclonal in origin[37]. On a cell-by-cell level, however, all tissues show evidence of genomic heterogeneity, which arises from insults that occur owing to the infidelity of DNA replication, imperfect DNA repair and other cellular processes[36,38]. In addition, and probably particularly relevant for prostate cancer, DNA damage occurring during transcription can further contribute to genomic diversity[39,40]. Therefore, every cell has a slightly different genetic make-up and can harbour numerous unique mutations[38,41,42]. However, most of these alterations do not affect cell growth and are therefore inconsequential. Neoplastic growth occurs when driver genes are altered such that cell fitness is increased. Most solid tumours are thought to arise from a single cell that gains neoplastic growth potential through accumulation of genomic and heritable epigenetic alterations that change the cellular phenotype[43]. Driver gene alterations are shared between all cells arising from the initiating cell. Therefore, daughter cells of this tumour-initiating cell, although they share the vast majority of genomic information with all other cells in the body, show distinct driver gene changes that are selected for, giving rise to neoplastic clonal expansion.

Within this clonal cell population, individual cells continue to accumulate additional gene alterations, resulting in subclonal expansions within the tumour mass that exhibit distinct genetic and phenotypic diversity[44]. A given mutation is considered subclonal if it appears in only a fraction of the tumour cells that otherwise share a set of founding mutations common to all of the cells in the tumour. The detection of a subclonal population is therefore dependent on an assay-specific technical detection threshold and a statistical threshold. More than half of the somatic alterations in an initiating cell are estimated to originate before neoplastic transformation and at least a subset of these changes are shared with adjacent benign cells[45]. Tracing experiments demonstrate that early non-driver alterations can occur in tissue-resident stem cells from which the glandular network of the prostate is populated[46]. These changes are distinct from the germline sequence found in other tissue types and can be detected in both benign and cancer tissues[25,45,46] (FIG. 1. This finding probably explains why cancer and adjacent benign tissue can often share genomic alterations and also provides an explanation for the high level of intra-tumoural heterogeneity seen in prostate cancer[25,47].

Microheterogeneity and macroheterogeneity.

The expansion of a tumour into a clinically detectable mass generates a vast range of genotypes with variable impacts on cell fitness. Given this high level of genetic diversity in tissues, the term heterogeneity needs to be used carefully and in the appropriate context. In particular, we need to consider which level of genetic heterogeneity is clinically relevant[36]. Microheterogeneity at the single-cell level is subjected to constant selection, which shapes the subclonal cell populations[43,48]. With the currently available technologies, microheterogeneity is challenging to study and is therefore of uncertain clinical significance at this point. The expansion of a single cell with unique favourable traits into a larger cell mass results in macroheterogeneity, which is more accessible using current methods (see below). Propagation of subclones can lead to complete overgrowth of other less well-adapted cell populations (clonal sweep) and results in further genetic diversification (FIG. 1. Studies applying multiregional sampling approaches, in which several regions of a single tumour mass are analysed, have documented that the vast majority of solid tumours comprise subclones with varying degrees of driver gene alterations[7,49–52]. On the basis of these studies, most solid tumours represent a mosaic of multiple tumour cell populations that have shared (truncal) and unique (branch) genomic and phenotypic features (FIG. 1). The level of subclonal diversity can vary between different tumours. Importantly, although in some tumour types increased intra-tumoural heterogeneity has been associated with worse clinical outcome, in other settings, tumours with very high subclonal diversity seem to be less aggressive, suggesting a complex relationship between the level of heterogeneity and clinical outcome[35,43,53,54].

The unique clonal constellation of prostate cancer.

Like most other tumour types, subclonal heterogeneity is common in prostate cancer[25,27,33,55]. Studies demonstrate that around 60% of tumour foci show evidence of multiple subclones[25,33,47,55]. Interestingly, these studies suggest that the subclonal diversity in a given index tumour focus is higher in primary samples than in metastatic samples. In addition, tumours with a more complex sub-clonal architecture have a higher rate of recurrence than monoclonal tumours[33,56]. Collectively, these data suggest that a high level of subclonal diversity in the primary tumour might increase the chance of metastatic dissemination. Given that the number of subclones capable of metastasis formation is probably very small, the clonal constellation in distant metastases of treatment-naive patients seems more homogeneous.

What sets prostate cancer apart from most other solid tumours is that the vast majority of primary prostate cancers occur multifocally[4,26,27]. This means that a diseased prostate gland harbours multiple topographically separate tumour foci. A large body of literature demonstrates that these distinct tumour foci show unique non-overlapping mutation profiles[4,25,27,29,40,57,58] (FIG. 1), suggesting that these tumours arise independently and follow separate evolutionary trajectories. Therefore, a given patient can harbour more than one genomically and phenotypically distinct prostate cancer (FIG. 2). These clonally independent tumours can show major biological differences and can contribute differently to disease progression and clinical outcome. The multiclonal nature of prostate cancer has important implications for clinical management and research. Therefore, improved

understanding of the driving factors involved in the multifocal nature should result in improved understanding of prostate cancer biology. For instance, it remains to be determined which endogenous or exogenous, epithelial cell intrinsic or microenvironmental factors contribute to this field cancerization and enable numerous independent tumour clones to arise in the prostate[59–61]. In addition, we need to determine in which clinical scenarios multiclonality is most relevant. To this end, defining methods that allow us to capture the heterogeneous nature of prostate cancer is important.

Studying genomic heterogeneity

In situ methods.

Genomic and phenotypic heterogeneity of complex tumour tissues can be assessed using two different methods: in situ approaches, which preserve the tissue architecture and enable cell-by-cell analyses and correlation with histomorphology features; and ex situ assays, in which the tissue is disrupted and extracted analytes can be measured using a variety of assay platforms. The most commonly used in situ methods in molecular pathology — immunohistochemistry and fluorescence in situ hybridization (FISH) — have been used extensively to study prostate cancer. For instance, the detection of *TMPRSS2-ERG* rearrangements by FISH, which occur in ~50% of prostate cancers, was applied to determine clonal relationships in the setting of multifocal primary tumours and metastatic disease[62,63]. In addition, FISH-based assessment of the tumour suppressor gene *PTEN*, which frequently shows subclonal copy-number loss, has contributed to our understanding of the order of genomic events occurring in prostate cancer[64]. However, FISH can be cumbersome to interpret and requires the use of fluorescence microscopy, thus limiting throughput and potential widespread clinical implementation.

The use of antibodies that can detect oncogenes or tumour suppressors by immunohistochemistry has greatly improved our ability to query genomic alterations directly in tissues. For instance, immunolabelling with antibodies to ERG has been shown to correlate closely with *ERG* rearrangement status and can therefore be used as a read-out to screen for ERG+ lesions[26,65,66]. Combination immunostains of ERG and SPINK1, which is expressed in a subset of ERG− tumours, can highlight clonally distinct tumour cell populations directly on standard pathology slides[67]. Similarly, antibodies to PTEN, p53 and RB1 can be used to highlight clonal and subclonal changes to these important tumour suppressor genes[68–70]. Therefore, genomic alterations can be visualized in the tumour tissue using surrogate immunohis-tochemistry markers that are strongly associated with certain genetic alterations (FIG. 2). Genetically validated clinical grade immunohistochemical assays, in particular for targets that have potential as clinical prognostic and predictive biomarkers such as *PTEN* and *TP53* alterations, represent extremely useful ancillary diagnostic tools that can easily be implemented in clinical practice[68]. The major advantages of such in situ methods is that they require a minimal amount of tumour tissue, allow correlation of molecular changes with tumour morphology and can be embedded in already existing workflows in clinical laboratories, ensuring rapid and robust results.

Sequencing approaches.

Although early evidence documenting the clonal heterogeneity of prostate cancer was drawn from the analysis of polymorphic microsatellite markers and comparative genomic hybridization of microdissected tumour foci[19,71–73], these technologies have been almost completely replaced by massively parallel sequencing approaches. With increasing accessibility to next-generation sequencing, numerous studies have investigated clonal and subclonal heterogeneity in prostate cancer by using multiregional sampling followed by whole-exome or whole-genome sequencing. All of these spatial genomics studies have in common that multiple tumour foci are macro- or micro-dissected and isolated DNA is subjected to sequencing. Extensive efforts in this space have provided definitive evidence that, in a subset of patients, individual tumour foci do not share any driver gene alterations and likely arose as independent clones, confirming the multiclonal nature of primary prostate cancers[27,47,57,74,75]. In addition, by using deep sequencing and clustering of variants by their mutant allele fraction, the presence of subclonal populations of cells can be inferred, providing insight into the temporal subclonal evolutionary relationships between different tumour regions[28,33,34,55,56,76]. Phylogenetic trees can be reconstructed that depict the clonal evolution of truncal and unique alterations[34,53,77]. Importantly, such analyses provide insights into the origin and timing of cancer evolution and have suggested that early driver gene alterations can occur decades before clinical manifestation and diagnosis[34,78,79]. Although these approaches allow inferential assessment of clonal evolutionary relationships and macroheterogeneity from bulk tumour sequencing data, they are limited by sequencing depth and the sensitivity of calling algorithms, which are prone to biases[48,56].

Single-cell sequencing and other emerging technologies.

Several single-cell analysis platforms have been developed to study microheterogeneity at the level of individual cells, and these tools have been applied in small-scale studies of prostate cancer[80–82]. Although technically challenging, these extremely powerful approaches have the potential to provide a more comprehensive picture of the diversity of tumour cell populations and enable precise delineation of the clonal dynamics in therapeutic response and emergence of resistance[83,84]. Such single-cell technologies enable detailed analysis of the cellular tumour composition, genomic alterations and epigenetic states[85,86]. However, these ex situ approaches rely on dissociation of tumours to single cells, which disrupts tissue architecture. Novel in situ approaches such as digital spatial profiling technologies permit the assessment of mRNA and protein expression by highly scalable next-generation sequencing read-outs directly from tumour tissue sections[87,88]. The great power of these in situ approaches lies in the preservation of tissue architecture, which allows the cross-referencing of molecular findings with detailed topographical and histomorphological information. Therefore, these tools will be perfectly complementary to single-cell sequencing platforms to understand intra-tumoural heterogeneity.

A major limitation of all of these sequencing-based technologies is that they mostly rely on the assessment of alterations at the RNA or DNA level. However, evidence is growing that transcriptomic, genomic and epigenetic studies are often not adequate for accurate prediction of protein expression[89,90]. Therefore, efforts have been focused on developing high-throughput proteomics approaches that permit the robust detection of protein

expression changes in clinical specimens, including formalin-fixed, paraffin-embedded samples[89–91]. Application of these approaches has already yielded important new insights into the proteogenomic heterogeneity of prostate cancer and will further improve our ability to determine biologically relevant alterations[92].

Primary prostate cancer

Anatomical considerations.

The prostate can be divided into distinct zones[93]. The peripheral zone, which constitutes the majority of the glandular tissue of the prostate, harbours most cancers, whereas the central and transition zones have a much lower rate of cancer incidence[59,94]. These anatomically defined zones show distinct gene expression profiles, and lesions arising in different zones show different propensities for certain genomic alterations[95]. For instance, *ERG* translocations are common in peripheral zone tumours but rarely seen in lesions arising from the transition zone[96]. Notably, racial differences have also been associated with distinct zonal distributions of tumours[97]. These findings suggest that the anatomical site and zone in which tumours arise might influence the spectrum of genomic alterations and potentially the tumour phenotype, providing evidence that tumour location can contribute to tumour heterogeneity.

The morphological spectrum of primary prostate cancer.

Primary prostate cancers can display a broad range of morphological features and growth patterns, and the appreciation that certain morphologies are associated with distinct patterns of molecular alterations and biological behaviour is increasing[98–101]. Although the grading system for prostate cancer has been revised several times over the past few decades, the Gleason score remains the most important risk assessment tool for clinical decision-making in localized prostate cancer. Its ingenuity lies in the integration of architectural heterogeneity into a composite score that captures the morphological heterogeneity of prostate cancer[98]. Importantly, several studies have shown that the number of genomic alterations and the frequency of driver gene changes increases in higher grade tumours[69,102,103]. Given the heterogeneity of Gleason patterns, the question arises whether morphologically distinct growth patterns in a given tumour focus (for example, Gleason pattern 3 versus Gleason pattern 4) are clonally related. Analyses demonstrate that at least a subset of synchronous adjacent Gleason pattern 3 and Gleason pattern 4 lesions are clonally linked and share initiating driver alterations but show subclonal branching evolution[104–108]. These findings indicate that Gleason pattern 3 and Gleason pattern 4 lesions can arise from a common ancestral clone but follow divergent genomic and morphological trajectories.

The multifocal nature of prostate cancer.

More than 80% of prostate glands harbouring cancer have two or more topographically and morphologically distinct tumour foci[19,109] (FIG. 3). The reported numbers of individual tumour foci in a given prostate are not very consistent, probably due to different definitions of a focus and the challenges in comprehensively sampling the entire prostate in an attempt to detect every tumour focus[110]. Further complicating the assessment of individual tumour foci is the complex growth pattern of primary prostate cancer, which lacks a well-defined

invasion front and shows diffuse infiltrative growth[98,110,111]. Whereas some tumour foci seem to collide and form large, morphologically indistinguishable tumour masses[111], other patients (as shown in FIG. 3) show completely non-contiguous tumour foci with distinct morphological features and histological grades[19,20].

Genomic and epigenetic evidence of multiclonal prostate cancer.

Although the multifocal nature of primary prostate cancer suggests a potential polyclonal origin, detailed genomic studies have only recently provided convincing evidence that the majority of topographically distinct tumour foci show non-overlapping mutation profiles, suggestive of an independent clonal origin[27,57,62,71,112,113]. Multiregional sampling and whole-exome sequencing demonstrated that tumour foci from 3 out of 4 patients with primary prostate cancer showed no overlapping genomic changes[57]. This finding was further corroborated by whole-genome sequencing of 23 distinct tumour regions in 5 patients, which showed that a given patient can harbour two separate prostate cancers that share no relevant genetic alterations[25]. In the largest study to date, 2–3 distinct tumour foci were analysed in 41 patients using whole-exome sequencing. Strikingly, 76% of multifocal tumours showed nonoverlapping mutations, including known driver gene alterations, in topographically distinct tumour foci[27]. Taken together, a large body of literature strongly supports the notion that multiple clonally independent lesions can be present in a diseased prostate gland.

In addition to genomic changes, DNA methylation alterations have been shown to co-segregate with copy-number changes, suggesting that genomic and epigenomic events can evolve on a shared clonal trajectory[114]. Studies analysing the pattern of DNA methylation alteration in multifocal prostate cancer revealed striking differences in methylation profiles, demonstrating that lesions with distinct genomic alterations also show unique methylation patterns[115–117]. These studies confirm that DNA methylation alterations can be used to infer clonal relationships and dynamics[114–116]. These observations pave the way for future studies, which should aim to understand the diversity of other epigenome marks in the context of multiclonal prostate cancer in greater detail[117].

Challenges of multiclonality

Challenges of assessing multifocality using biopsies.

Assessing the full clonal and subclonal heterogeneity of prostate cancer is theoretically possible when evaluating a radical prostatectomy specimen. However, multifocality and multiclonality represents a major challenge for prostate cancer diagnosis on needle biopsy samples. Using a dual immunohistochemistry approach for ERG and SPINK1, it was shown that ~25% of biopsies with noncontiguous core involvement sample at least two clonally independent tumours, with the remaining 75% sampling the same tumour going in and out of the plane of section of the biopsy[118]. This finding highlights the complexity of assessing multifocal or multiclonal disease on core biopsies but also shows that simple immunohistochemical tools provide robust and cost-efficient ways of assessing clonality on standard diagnostic biopsies. Although very powerful for research studies, comprehensive immunohistochemical evaluation of all tumour-containing biopsy samples in an attempt to

catalogue clonal heterogeneity is not feasible for routine clinical use. Using a targeted sequencing approach, the heterogeneity of genomic alterations in biopsy samples and matched radical prostatectomy samples has been assessed. On average, only 19% of the mutations detected in the prostatectomy specimen were represented in matched preoperative biopsies[119]. Although technical improvements can potentially refine biopsy sampling, this finding implies that systematic needle biopsies are probably inadequate for detecting all relevant spatially and molecularly distinct tumour areas. This observation has important implications for clinical practice in which primary tumour samples are often used to make decisions about actionable alterations in distant metastases[120]. Given the high level of clonal heterogeneity, primary tumour samples need to be selected carefully for genomic studies and, in some patients (where possible), multiregional sampling or direct biopsy of the metastatic lesion should be performed.

Undersampling of high-grade lesions with aggressive molecular features is of particular concern in the setting of active surveillance. For instance, in patients in whom clonally and molecularly distinct tumours with different biological potentials to metastasize can coexist, undersampling of a more aggressive lesion might cause treatment delays and loss of curative opportunities[121]. Multiparametric MRI, which integrates perfusion, diffusion and anatomical imaging, has greatly improved the accuracy of image-guided tumour mapping[122]. The widespread use of MRI-guided biopsies has improved the detection rate of high-grade, clinically significant cancers and improvements in imaging approaches are expected to further augment the accuracy of diagnosis[123,124]. One of the greatest opportunities for MRI and other functional imaging approaches lies in the ability to cross reference in vivo imaging data with molecular tumour features. The identification of imaging characteristics associated with a high risk of clinically significant disease that are functionally linked to defined molecular alterations will eventually enable the detection of molecular intra-tumour heterogeneity and provide an exciting new intersection between radiology and pathology[125]. Paired with molecular imaging, these approaches will greatly improve tissue sampling and augment the accuracy of precision medicine in localized prostate cancer[123,126].

Multifocality as a barrier for biomarker development and implementation.

The multiclonal nature of prostate cancer represents a leading factor contributing to the underperformance of current diagnostic paradigms and molecular biomarkers. For instance, in patients with comprehensive profiling of all tumour-containing material, high degrees of heterogeneity of DNA ploidy and *PTEN* status were noted in up to 75% of patients[18]. Along these lines, the high level of molecular heterogeneity poses a major challenge for the implementation of commercial gene expression assays that are aimed at guiding clinical decision-making[127,128]. Although these expression signatures provide prognostic information and correlate well with tumour grade within a sample, patients with multifocal prostate cancer harbouring both high-grade and low-grade lesions represent a challenge. Expression signatures are usually determined from a single biopsy core that shows the highest Gleason grade. However, risk scores can vary greatly between different tumour foci, and the assessment of a single focus might not capture the complex heterogeneity present in primary tumours[129]. In addition, in patients with multifocal prostate cancers, analysis of a lower grade component cannot be extrapolated to adjacent high-grade lesions[130]. Therefore,

regardless of the downstream analysis, sampling of different tumour lesions in the setting of multifocal prostate cancer remains a major challenge. The increased use of neo-adjuvant therapies for high-risk patients has provided a unique opportunity to delineate resistance mechanisms and analyse the clonal and subclonal dynamics in primary tumours following androgen deprivation therapies[131]. For instance, a 2020 study revealed significant differential responses to systemic therapies in clonally and morphologically distinct tumour foci in the primary tumour and therefore highlights the relevance of inter-focal heterogeneity for clinical care[132].

Molecular definition of the index lesion.

Molecular studies have suggested that even small well-differentiated lesions can seed distant metastases[29,133]. For instance, our group tracked the origin of the lethal metastatic cell clone to a 2.2-mm well-differentiated (Gleason pattern 3) component in the primary tumour in a patient who died of prostate cancer. This lesion harboured a large number of the driver gene changes present in the distant metastasis including mutations in *TP53, PTEN* and *SPOP*, which were distinct from the surrounding primary tumour mass[29]. Although further studies are needed, this observation calls into question the concept of the index lesion as the tumour nodule defined by size and grade and suggests that molecular characterization of such lesions could provide additional insights into what should be considered the dominant clone[29,134]. This could be particularly relevant for focal therapy approaches that intend to ablate index lesions in a targeted manner to reduce the morbidities ensuing from other forms of treatment for localized disease[135]. However, it is important to note that in our study this well-differentiated component was present within a larger lesion that was a Gleason score 4 + 3 = 7 tumour[29]. Although metastases from an isolated Gleason score 3 + 3 = 6 tumour are exceedingly unlikely[136], evidence suggests that a Gleason pattern 3 component that is present contiguously with a Gleason pattern 4 tumour (3 + 4 = 7 or 4 + 3 = 7) is molecularly different from a Gleason pattern 3 that is only part of a Gleason score 3 + 3 = 6 tumour[137,138].

Regional lymph node metastases

Metastases to the lymph nodes (which can be detected in a subset of men at the time of radical prostatectomy) have been associated with adverse prognosis[139]. The prevailing hypothesis to explain this association suggests that distant metastases are seeded by lymph node metastases. However, clinical data show that ~30% of men with prostate cancer metastatic to regional lymph nodes are free from recurrence at 10-year follow-up even if left untreated after surgery[140]. In addition, mounting molecular evidence suggests that in different tumour types, draining lymph nodes represent stopping points rather than spring boards from which more distant metastases are launched[141]. For instance, in colorectal cancer, phylogenetic reconstruction of clonal relationships between primary tumours, lymph node metastases and distant metastases showed that in 65% of cases, lymphatic and distant metastases arose from independent subclones in the primary tumour[142]. In prostate cancer, the genomic relationship of matched primary tumour, lymph node and distant metastases has not been systematically studied and only limited data exist on the clonal relationship between primary and metastatic lesions. In a case report of a patient who died of metastatic

prostate cancer, we showed that a lymph node metastasis that was present at the time of initial presentation showed no overlap in driver gene alterations with the lethal metastases sampled at the time of death[29]. In addition, a study comparing copy-number alterations of primary prostate tumours and pelvic lymph node metastases showed that in a substantial fraction of cases (23.3%) the index primary tumour was not clonally related to the locoregional lymph node metastases[133]. Furthermore, clonal analyses suggest that lymph node metastases often originate from evolutionarily advanced subclones located at the periphery of the prostate gland, in a process that seems to be distinct from clones seeding osseous metastases[143]. Collectively, these studies suggest that regional lymph node metastases can arise from clonally distinct tumour cell populations and do not necessarily contribute to progression to metastatic disease.

Distant metastatic disease

Processes shaping the clonal constellation in metastases.

The fitness of a clone to form metastases is not only determined by its ability to shed cells into circulation but also by its capability to colonize, expand and eventually develop resistance to systemic therapies[144–146] (FIG. 1). In prostate cancer, metastases can take decades to emerge after initial diagnosis of localized disease[12,15,147]. However, once metastases are established, disease progression is greatly accelerated, suggesting that the early phases of metastasis formation represent a major bottleneck[6]. Large-scale analyses across solid tumour metastases suggest that the vast majority of metastatic lesions show subclonal homogeneity[148]. In particular, analyses of treatment-naive metastases have shown that the majority of driver gene alterations are shared between different metastatic sites[36,149]. By contrast, however, other reports provide evidence of early branching evolution during metastatic dissemination and extensive subclonal heterogeneity in pan-cancer analyses. These findings suggest the presence of widespread and potentially clinically relevant phenotypic and genomic differences between different metastatic sites[28,30,77,150]. This strikingly divergent assessment of the clonal composition of distant metastases highlights the need for additional studies in this field and probably reflects major differences in the analytical approaches and study cohort characteristics. For instance, the vast majority of studies demonstrating clonal homogeneity have used bulk tumour sequencing of a single metastatic site, whereas studies showing heterogeneity used deep-sequencing approaches and sampled multiple metastatic deposits[28,30].

Treatment history might also influence the genomic composition of a tumour. Systemic cancer therapies exert a strong selection pressure that can shape the clonal constellation of a tumour[151] (FIG. 1). After therapy, residual tumours might exhibit an altered clonal composition with newly emerging subclones that harbour alterations conferring resistance[51,152]. Particularly relevant for prostate cancer therapy is the crucial dependence of prostate cancer cells on the androgen receptor (AR) as a core lineage oncogene. Therapies that block AR signalling, which were arguably among the first targeted therapies in oncology, remain the mainstay of therapy for metastatic disease[153]. However, resistance to AR blockade and progression to metastatic castration-resistant prostate cancer (mCRPC) emerges in almost all patients[153]. Resistance to androgen deprivation is often associated

with genomic alterations of the *AR* locus, including copy-number gains and gain-of-function mutations. Although very common in mCRPC, these changes are almost never detected in hormone-naive patients, highlighting the strong association between certain genomic alterations and the patient's treatment history[56,154]. Second-generation therapies that further suppress either ligand synthesis (for example, abiraterone acetate) or binding to AR (such as enzalutamide) have been shown to be effective in suppressing AR signalling further but can select for clones that show resistance mechanisms that bypass the need for AR signalling for cancer cell growth[155]. Notably, AR signalling has been shown to regulate a number of key DNA repair genes and AR inhibition is associated with decreased DNA repair activity[156,157]. Therefore, blockade of the AR signalling axis could potentially result in decreased genomic integrity and contribute to intra-tumoural heterogeneity. More broadly, the contribution of AR blockade to the emergence and selection of subclones with *AR* alterations, and the potential interference with DNA repair activity, provides a rationale for a combination of AR-targeted therapies and other cytotoxic treatments early in the progression of high-risk patients[158].

Morphological and phenotypic heterogeneity in mCRPC.

Metastatic prostate cancer has a wide spectrum of histomorphologies, including features commonly seen in adenocarcinomas such as acinar, cribriform, single-cell and solid growth patterns, but also morphologies consistent with poorly differentiated or anaplastic carcinomas and neuroendocrine differentiation (carcinoid-like pattern and small-cell carcinoma)[159–162] (FIG. 4). Morphological patterns can vary greatly between different patients and also show a high level of inter-tumoural heterogeneity within an individual patient. Different morphologies can be present even at a single metastatic site[159–162] (FIG. 4g). These diverse histomorphological features suggest profound differences in cellular architecture, metabolism and epigenetic programmes. Although evidence exists that some tumour morphologies are associated with distinct expression profiles, additional studies are needed to investigate the relationship between histomorphological features and the underlying molecular alterations[31,163].

In contemporary mCRPC cohorts, the majority of metastatic lesions retain some level of prostatic lineage differentiation characterized by the expression of prostate epithelial markers such as AR, NKX3.1 and PSA[154,155,159,160,163]. However, treatment-associated changes in cellular differentiation have emerged as an important resistance mechanism to AR-directed therapies[31,155]. Loss of prostate epithelial marker expression (in particular AR) and in some cases gain of neuroendocrine differentiation have been observed in mCRPC[155,163]. These observations suggest that in response to therapy a subset of tumours can show alterations in prostatic lineage commitment and an increased phenotypic plasticity[155,164]. On the basis of these findings, metastatic prostate cancers can be classified into subgroups, defined by the expression of AR, AR target genes and neuroendocrine differentiation markers. In addition to tumours with high AR activity, other lesions show low or no AR activity in the absence of neuroendocrine marker expression (double-negative prostate cancers), co-expression of AR and neuroendocrine markers (amphicrine or adenoneuroendocrine prostate cancer) or show loss of AR expression and gain of neuroendocrine differentiation (neuroendocrine prostate cancer, NEPC), with the added

complexity of tumours transitioning between these defined phenotypes[155,163]. As variation exists even within these molecularly defined groups, further studies will probably result in additional refinement and subclassification.

Owing to its more aggressive biological behaviour, NEPC has been the focus of numerous studies over the past few years[164]. NEPC is characterized by loss of AR activity and the expression of neuroendocrine markers such as synaptophysin, chromogranin A, CD56 and INSM1, along with morphological features of small-cell carcinoma or high-grade neuroendocrine carcinoma[162,165,166]. The study of NEPC is complicated by the focal presence of neuroendocrine differentiation immunohistochemically in 'usual' acinar prostate cancer without any clinical relevance[162]. Recognition is also growing of hybrid tumours that have features of transdifferentiation from usual to neuroendocrine phenotype. At the molecular level, NEPC is enriched in genomic alterations in *RB1* and *TP53* but also displays striking epigenetic changes, suggesting that NEPC might evolve through subclonal epigenome evolution[31,164,167]. In cohorts of patients with advanced mCRPC, evidence for NEPC can be found in up to 15–20% of cases[155,161,167]. However, owing to differences in the clinical and treatment history, and the criteria applied to define neuroendocrine transdifferentiation, some variability exists in the frequency of NEPC reported in different mCRPC cohorts[154–161]. Therefore, although the subclassification of mCRPC will probably be of great relevance for clinical trial design and ultimately clinical practice, a set of consensus molecular markers and morphological features that enable the distinction of these phenotypic subgroups needs to be defined. To this end, a carefully selected set of biomarkers will need to be validated across different institutions.

Monoclonal origin of lethal distant metastases.

Given the multifocal and multiclonal nature of primary prostate cancer and the often heterogeneous phenotypes observed in anatomically distinct metastases, the question arises whether multiple clones of the primary tumour contribute to the distant metastatic disease burden upon metastatic dissemination (polyclonal seeding) or whether only one primary clone seeds all distant metastases (monoclonal seeding)[168]. Early studies focusing on the analysis of single gene alterations provided evidence for the monoclonal origin of distant metastases[63,169,170]. More recently, several whole-exome and whole-genome sequencing studies have demonstrated that anatomically distinct distant metastases share a large number of genomic alterations, confirming that most likely a single clone in the primary tumour gives rise to all distant metastases[28–30,114,171–173] (FIGS 1,5).

Homogeneity versus heterogeneity.

Analyses of multiple metastatic deposits at the genomic, epigenetic and transcriptomic levels from men who died of prostate cancer have revealed a high concordance in driver genomic and epigenetic alterations between different metastatic sites in a given patient[114,172,173]. These findings, which are all based on bulk sequencing and array-based assays, demonstrate limited inter-tumoural differences, which probably do not greatly affect clinically relevant tumour phenotypes[114,172]. However, deep sequencing combined with bioinformatic reconstruction of subclonal architectures has revealed more complex patterns of subclonal diversity between different metastases from distinct anatomical sites[28,30]. These studies have

demonstrated that individual subclones can seed from one metastasis to another in a polyclonal fashion. Therefore, every metastatic site can be considered a conglomerate of different subclones (FIG. 5). For instance, *AR* alterations are rarely truncal and different subclones show distinct *AR* alterations, suggesting convergent evolutionary processes that activate AR signalling[28]. Detailed phylogenetic reconstructions demonstrate that independent subclones in the primary tumour or metastases can seed to the same metastatic site in distinct waves, indicating that early metastatic dissemination can potentially pave the way for future seeding events[28,30]. This finding is clinically relevant, as it supports the notion that surgical removal of the primary tumour or ablation of oligometastatic tumour deposits can potentially eliminate sources for additional seeding events and therefore result in improved oncological outcomes in patients with metastatic disease[174,175].

Although the number of patients included in these detailed sequencing studies is currently low, it is of interest that the extent of shared truncal genomic alterations between different subclones present in a given patient can vary greatly (range 40–90%)[28]. An intriguing hypothesis potentially explaining this observation is that truncal mutations in some patients might confer a maximum level of fitness such that additional mutations do not result in further improvements. Other tumours, however, might start out with a founder clone with limited fitness, necessitating the additional accumulation of driver alterations, thus resulting in increased subclonal diversity[48]. In support of this notion, a study demonstrated that tumours have less genomic subclonal heterogeneity as patients progress towards NEPC in later stages of the disease[167]. Therefore, clinically more aggressive disease can potentially appear clonally more homogeneous with a high level of shared genomic and epigenetic alterations[167]. This finding emphasizes the importance of the timing of sampling and suggests that as subclones evolve, the fittest subclonal population can take over the majority of the tumour burden. Such clonal sweeps will result in more homogeneous cell populations (FIGS 1,5). In earlier stages of tumour progression, however, a higher level of subclonal heterogeneity might prevail. Through the passage of clonal bottlenecks, this heterogeneity will become more homogeneous. Therefore, longitudinal sampling efforts are necessary to capture the dynamically changing genomic and epigenomic features of mCRPC[167,176].

Assessing inter-tumoural heterogeneity in mCRPC.

Historically, the complex heterogeneity of metastatic disease has been difficult to study, mostly owing to major challenges in obtaining samples from multiple metastatic sites. In addition, comprehensively sampling bone lesions remains a major challenge. A growing number of studies have assessed the molecular alterations in mCRPC from biopsy specimens[154,177–179]. However, many of the current insights into the biology of mCRPC were derived from rapid autopsy studies, in which multiple metastases are sampled at the time of death[28,155,159,160,172,173]. One limitation of these studies is that they only provide end-point assessments of heavily pretreated patients and might therefore not be representative of earlier stages of the disease. In addition, most genomic assessments of mCRPC have been restricted to bulk sequencing of tissue samples and data are currently limited on microheterogeneity in mCRPC tissue. However, single-cell sequencing efforts of circulating tumour cells from patients with mCRPC have revealed diverse copy-number profiles, suggesting a high level of genomic heterogeneity at the single-cell level that is not

adequately captured by analyses of matched biopsy material[180]. Given that the level of microheterogeneity is proportional to the size of the metastatic lesion, bulky metastases have an increased chance of harbouring intrinsically resistant cells. This notion provides a rationale for the use of cytoreductive therapies earlier in disease progression[36]. Indeed, several trials have reported improved outcomes in patients with prostate cancer when hormonal or chemotherapeutic agents were introduced at earlier stages of the disease[158,181].

Most studies assessing inter- and intra-tumoural heterogeneity in mCRPC have focused on genomic alterations[28–30,171–173]. However, interest is growing in profiling epigenome changes and genome-wide chromatin interaction profiles of AR and other key transcription factors in advanced prostate cancer[182,183]. Epigenetic modifications have been implicated as key drivers of phenotypic plasticity, and the evolution of tumour subclones is probably defined by interactions between genetic and epigenetic factors[184]. Studies assessing epigenetic intra-tumoural heterogeneity have thus far primarily focused on DNA methylation alterations and have demonstrated that despite an overall high level of clonal conservation of DNA methylation alterations between different metastases, evidence exists for divergent epigenome alterations during tumour progression[31,114,167]. The potential reversibility of epigenetic changes and the expanding portfolio of drugs that target epigenetic pathways should encourage future research in this field[183].

Transcriptional profiling of autopsy specimens has revealed a strong overlap in transcriptional programmes in anatomically distinct metastases[114,172]. Similarly, phosphoproteomic analyses have demonstrated high inter-individual, but limited intra-individual, heterogeneity at the level of kinase pathway activation[185]. These studies suggest the conservation of transcriptional and post-translational protein modification patterns across different metastases in most patients with end-stage mCRPC[172,185]. However, a study investigating the transcriptomic profiles of multiple metastatic sites in 34 patients revealed 5 cases (14.7%) of substantial inter-tumoural heterogeneity at the transcriptomic level[163]. Inter-tumoural differences have also been observed at the protein expression level for AR and RB1 in a subset of patients[186,187]. Collectively, these results suggest that although the vast majority of mCRPC lesions in a given patient might show overlapping molecular characteristics, a subset of patients exhibit inter-tumoural heterogeneity in pathways that are relevant for prostate cancer therapy. Notably, however, mRNA and protein-based assays, in particular, are susceptible to pre-analytic changes, which can potentially contribute to major differences in analyte abundance between samples for different anatomical sites. Therefore, RNA sequencing and immunohistochemical data need to be interpreted with this potential bias in mind.

Clinical challenges of heterogeneity in mCRPC.

During the clinical management of mCRPC, different subclones can emerge and disappear, depending on the effectiveness of therapies[167,188,189]. As described for other tumour types, subclonal evolution can be greatly influenced by therapy[24,36,43,51]. Every effective therapeutic intervention will restrict and alter the clonal composition of a tumour, eventually resulting in the emergence of resistant clones that can withstand the selection pressure imposed by therapy. Small populations of pre-existing therapy-resistant subclones can re-

populate the entire tumour burden, even if the vast majority of tumour cells are sensitive to the therapy[24,190]. In colorectal cancers, for instance, pre-existing low-frequency mutations in *KRAS* are strongly selected for and contribute to resistance to EGFR blockade[190]. In addition, adaptive epigenome changes are thought to contribute to the resistance phenotype[31,191]. Therefore, therapy resistance is probably driven by an interplay of the selection of pre-existing cell populations and de novo adaptation processes[24].

A body of literature supports the notion that truncal alterations predict immediate response to targeted therapies, whereas subclonal alterations can be a major driver of resistance that emerges in response to therapy[24,36,190]. Although the assessment of a single tumour tissue biopsy sample might miss a subset of alterations, the implications for initial treatment decisions are probably limited. Importantly, genomic alterations that are associated with initial clinical responses to targeted therapies in prostate cancer, such as mutations in DNA repair genes (including *BRCA2* and *ATM*) and mismatch repair genes, have been shown to be truncal and shared between different metastatic sites[56,154,172,192]. Therefore, a single biopsy sample should be adequate for determining a given patient's mutation status for such truncal events. On the other hand, a single biopsy sample will probably be insufficient to monitor subsequent resistance mechanisms that emerge in response to targeted therapies[193]. Particularly relevant for prostate cancer are alterations that change the response to AR-targeted therapies, including genomic changes of the *AR* locus, which have been shown to occur in a subclonal manner[188].

Apart from genomic biomarkers, interest in assessing morphological and other phenotypic changes in mCRPC biopsy tissues is increasing. Given the inter-tumoural heterogeneity in histomorphological features and transcriptomic findings outlined above, a single biopsy might not capture these features adequately. This problem is highlighted by a study that showed discrete regions of NEPC and adenocarcinoma even within the same core needle biopsy in 7 out of 27 patients with NEPC (26%)[161]. As the presence of neuroendocrine phenotypes has been shown to be associated with a lack of response to AR-targeted therapies, identifying such heterogeneous cell populations early during disease progression is important[161,167].

Delineating longitudinal changes.

mCRPC is a dynamic disease necessitating the development of tools for longitudinally monitoring the evolving tumour burden. Serial metastatic tumour biopsies are invasive for patients, technically challenging and do not allow (as described above) a comprehensive sampling of all metastatic sites. Therefore, alternative methods that capture tumour heterogeneity in a less invasive and cost-effective manner are needed. Liquid biopsy strategies, which involve the assessment of circulating plasma cell-free DNA (cfDNA), circulating tumour cells and other analytes, have been developed over the past few years and applied to mCRPC[176,194,195]. Genomic analyses of cfDNA have been used to investigate the dynamic evolution of *AR* alterations and provided evidence that resistant subclones can be detected in circulation even before clinical evidence of disease progression under therapy[188,189,196]. cfDNA-based biomarkers can therefore potentially expand the critical

window for therapeutic intervention and provide valuable information on subclonal dynamics[167,176,188,190].

Complementary to blood-based assays, functional imaging applications can capture inter-tumoural heterogeneity in mCRPC at the macroscopic level[197,198]. For instance, a retrospective series showed heterogeneous imaging findings in response to systemic therapies in up to 40% of patients and the study documents the feasibility of using molecular imaging to decipher functional inter-tumoural heterogeneity[197]. Integration of these new technologies will be increasingly important in clinical practice and will ultimately improve patient care.

Conclusions

Over the past decade, our understanding of the molecular and phenotypic heterogeneity in prostate cancer has matured. Through extensive profiling studies we have learned about the complex constellation of genomic diversity in primary tumours and metastases. In parallel, methods for delineating the heterogeneity of molecular alterations have greatly improved and become more accessible. The clinical challenges posed by the complex molecular heterogeneity of prostate cancer will require a multidisciplinary approach. Insights from imaging and molecular pathology studies will need to be paired with careful clinical annotations to establish features of clinically relevant heterogeneity. In addition, novel computational approaches and deep learning algorithms will be necessary to analyse the resulting multidimensional data. Ultimately, the integration of different levels of information that capture disease heterogeneity will provide an in-depth understanding of tumour biology and will allow us to overcome obstacles to precision medicine posed by intra-tumoural heterogeneity.

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Glossary

Inter-patient heterogeneity

Differences in tumour genotypes and phenotypes between individual patients.

Intra-tumoural heterogeneity

Genomic, epigenetic, transcriptomic and phenotypic differences within a tumour mass.

Inter-tumoural heterogeneity

Differences between anatomically distinct tumour sites within a given patient.

Multifocality

Spatially distinct and often histomorphologically different tumour lesions within one affected organ.

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Key points

- Primary prostate cancers are often multifocal with spatial and morphologically distinct tumour foci.

- Individual tumour foci can show non-overlapping truncal genomic alterations, suggesting that multiple clonally distinct cancers can arise in a given patient.

- Intra-tumoural and inter-tumoural heterogeneity present within the prostate gland poses diagnostic challenges.

- Despite the multiclonality of primary cancer, clonal bottlenecks imposed by the metastatic process and further by therapeutic interventions seem to select for a single dominant clone in lethal metastatic prostate cancer.
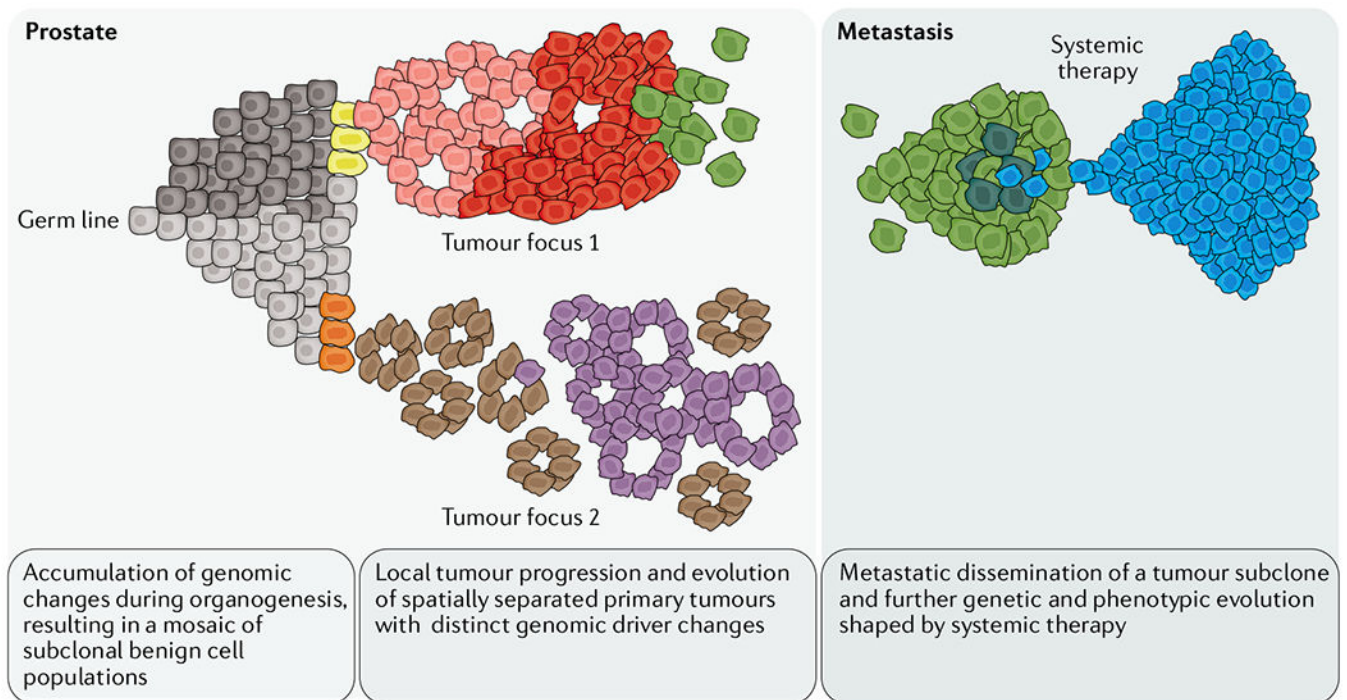
Fig. 1 |. Model of clonal progression of prostate cancer.
In the context of benign epithelial cells, which can harbour unique genomic alterations, precursor lesions can arise and progress into clonally and spatially distinct foci of invasive carcinoma. Within a given tumour focus, individual cells can acquire additional genomic driver changes, resulting in subclonal tumour cell populations. Subclones can further evolve, replace other tumour cell populations (clonal sweep) and disseminate to distant sites. Disseminated tumour cells can clonally expand, but systemic therapies can induce a major re-sculpting of the subclonal composition selecting for cells with intrinsic or adapted resistance mechanisms. Resistant subclones that pass through therapy-induced clonal bottlenecks contribute to disease recurrence and therapy failure.
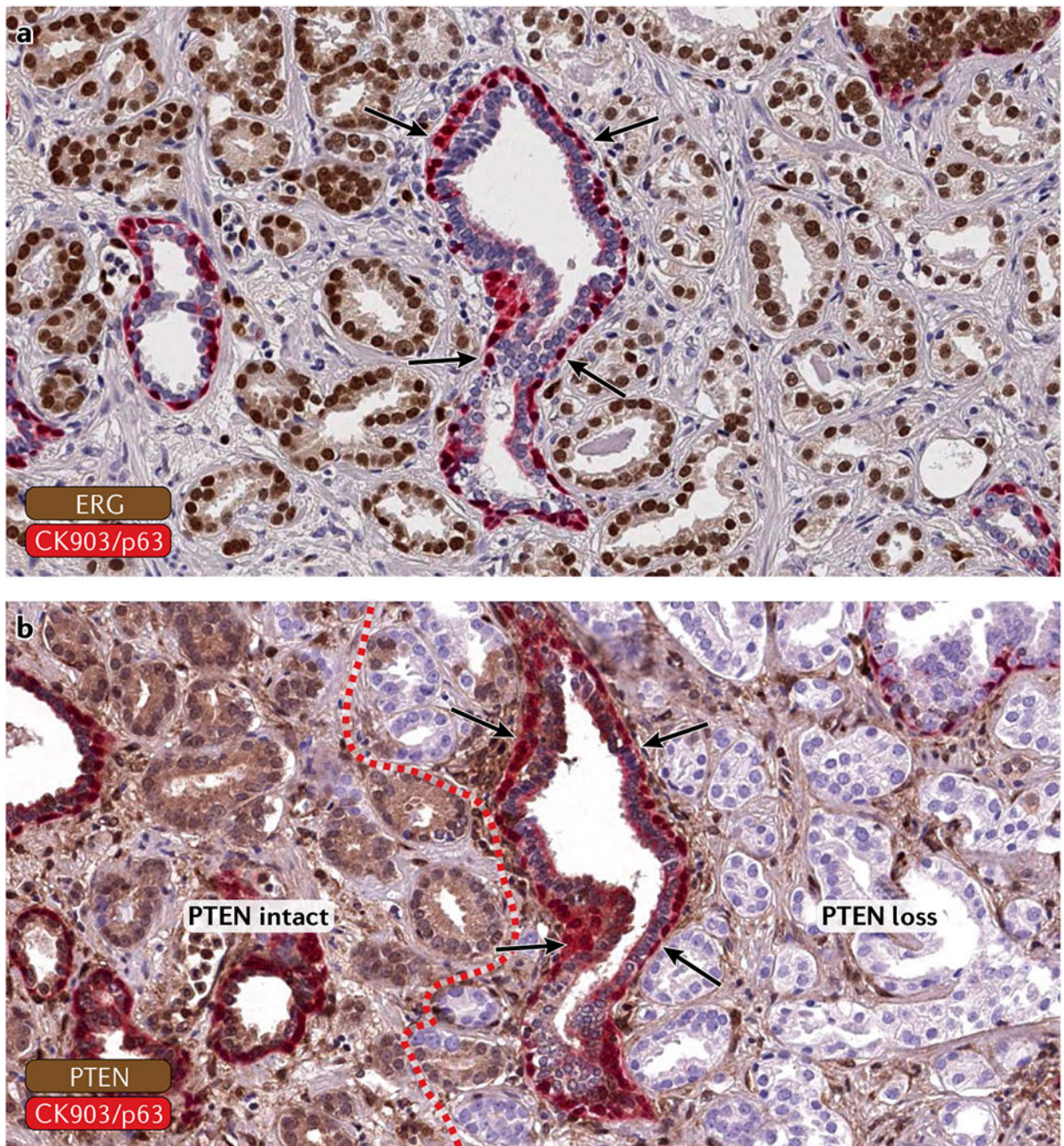
Fig. 2 |. Visualizing clonal and subclonal heterogeneity in tumour tissues.
Genetically validated antibodies for ERG and PTEN can be used to highlight clonal and subclonal cancer ceLL populations in tissue sections. **a** | An ERG+ (brown nuclear stain) tumour that infiltrates between benign glands (highlighted by an intact basal ceLL Layer, red stain). Arrows show a benign gland. **b** | The tumour shows subclonal loss of PTEN (loss of cytoplasmic staining) in a subset of cancer glands (separated by the red dotted line). Intact basal cells are shown in red. Arrows show a benign gland.
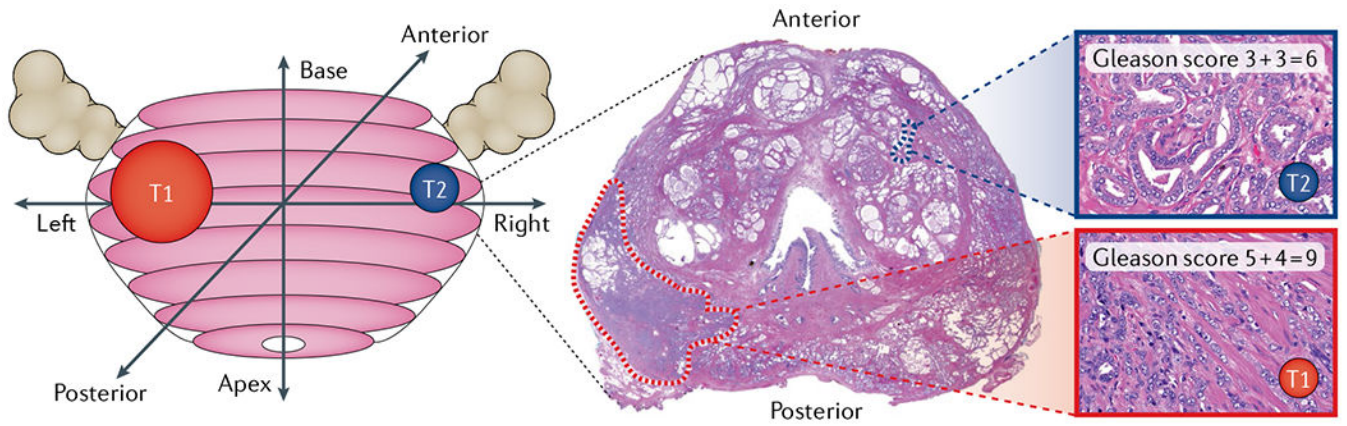
Fig. 3 |. Multifocal prostate cancer.
Reconstruction and whole-mount cross-section of a radical prostatectomy specimen with two distinct tumour foci. Note that the larger tumour focus located in the left posterior prostate shows high-grade morphology and extraprostatic extension (Gleason score 5 + 4 = 9), whereas a smaller anterior tumour appears well differentiated (Gleason score 3 + 3 = 6).
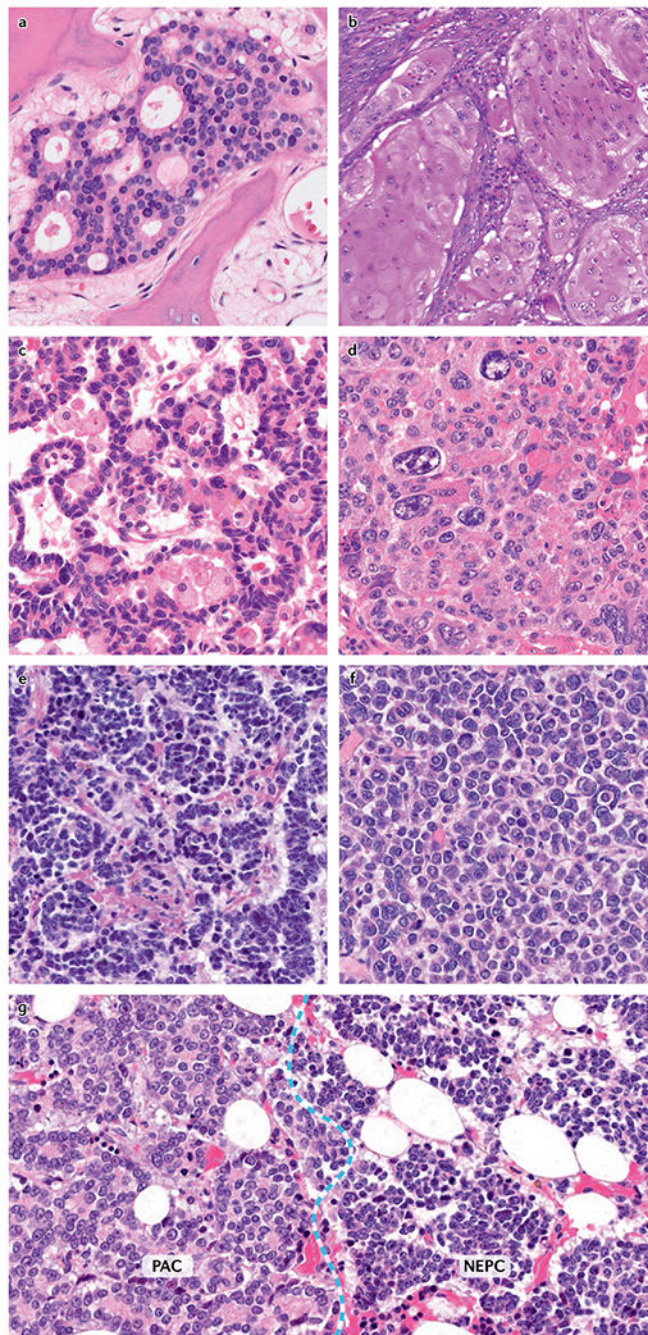
Fig. 4 |. Morphological heterogeneity in mCRPC.
a | Adenocarcinoma with cribriform architecture. **b** | Adenocarcinoma with squamous differentiation. **c** | Carcinoid-like differentiation in a neuroendocrine-marker-positive carcinoma. **d** | Poorly differentiated carcinoma with pleomorphic giant cells. **e** | Small-cell carcinoma. **f** | High-grade carcinoma with discohesive cells. **g** | Hybrid lesion composed of prostatic adenocarcinoma (PAC) with cribriform morphology (left) and small-cell neuroendocrine prostate cancer (NEPC) (right). mCRPC, metastatic castration-resistant prostate cancer.
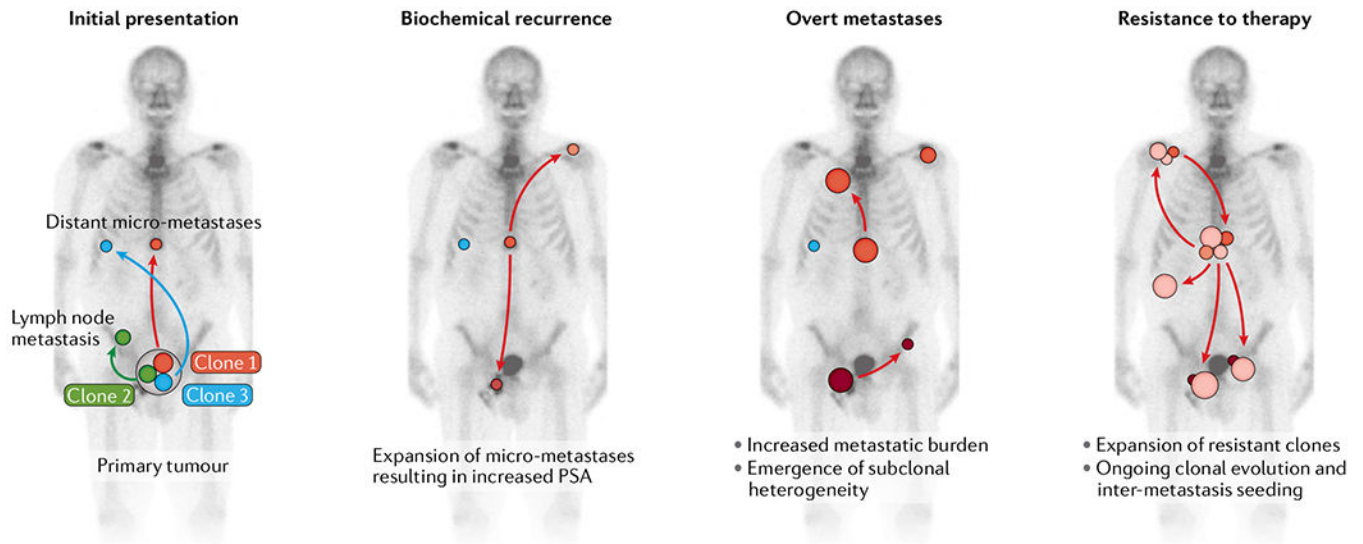
Fig. 5 |. Schematic of scenarios of clonal evolution of metastatic prostate cancer.
Initial presentation: primary prostate cancers often harbour more than one tumour clone. Distinct clones can have different metastatic potential. Although clone 1 and clone 3 can seed distant micro-metastases, clone 2 is restricted and cannot progress beyond the formation of nodal metastases. Biochemical recurrence: after surgical resection of the prostate, the expansion of micro-metastatic tumour deposits results in increased PSA levels. Note that at this stage, it is possible that more than one clone from the primary tumour can contribute to the pool of the distant micro-metastases. Although some clones expand and seed additional metastases (clone 1), other clones stay dormant or show minimal expansion (clone 3). Overt metastases: as the size of individual metastases increases, the subclonal compositions of the tumours broaden and additional metastases are seeded. Resistance to therapy: systemic therapy results in a re-sculpting of the clonal composition of distant metastases. Clone 3 and several subclonal lesions of clone 1 are effectively eliminated or greatly reduced in their size. A new subclone that arose from clone 1 is therapy resistant, expands and further disseminates. Individual subclones that arise in distinct metastases seed to other sites (inter-metastasis seeding), thereby contributing further to the inter-tumour and intra-tumour heterogeneity.