Clonal origin and spread of metastatic prostate cancer

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Abstract

Metastatic disease is responsible for the majority of prostate cancer deaths. The standard treatment for metastatic disease is surgical or chemical castration in the form of androgen deprivation therapy. Despite initial success and disease regression, resistance to therapy ultimately develops and the disease transitions to castration-resistant prostate cancer, which is uniformly fatal. Thus, developing an understanding of genetic evolution in metastasis and in response to therapy has been a focus of recent studies. Large-scale sequencing studies have provided an expansive catalog of the mutation events that occur in the prostate cancer genome at various stages of disease progression. Small-scale studies have interrogated the genomic composition of multiple metastatic sites within individual patients or have tracked clonal evolution longitudinally in tissues, circulating tumor cells, or circulating tumor DNA. Collectively, these efforts have provided a new conceptual framework for understanding the origin of prostate cancer, as well as the origin and evolution of metastatic disease. In this review, we highlight these recent insights into the spatiotemporal landscape of genetic evolution of prostate cancer.

Key Words

- metastasis
- prostate
- endocrine therapy resistance
- cell lineage/genetics
- clonal spread

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Introduction

Prostate cancer is the most frequently diagnosed cancer in men and accounts for an estimated 142,000 deaths in eveloped countries each year (Torre *et al.* 2015). Five-year survival rates for localized diseases are high (>90%) because localized cancers can be treated successfully with surgery and radiation. In normal prostate cells, the androgen receptor (AR) functions as a master transcriptional regulator activated by the androgens testosterone and dihydrotestosterone. Accordingly, prostate cancer presents as an androgen and AR-dependent disease (Dehm & Tindall 2011). For patients in whom surgery and radiation are not curative, the standard systemic treatment is functional suppression of AR transcriptional activity through androgen deprivation therapy (ADT). ADT includes surgical castration, pharmacological castration, and antiandrogen therapy (Dehm & Tindall 2011). ADT is initially successful in most patients; however, over time, castration-resistant disease develops. Therefore, development of therapy resistance and transition to castration-resistant prostate cancer (CRPC) are major clinical challenges. CRPC is characterized by rising PSA levels (signifying reactivation of AR transcriptional activity), an increase in tumor size, and metastatic spread (Knudsen & Scher 2009). Second-line ADT drugs that provide a more effective blockade of systemic androgen synthesis (abiraterone acetate) or act as higher-affinity antagonists of AR (enzalutamide) improve overall survival (Beer *et al.* 2014, Ryan *et al.* 2013).

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However, in many patients, second-line ADT fails to achieve control of tumor growth, which indicates frequent occurrence of primary resistance. Additionally, secondary resistance to these therapies inevitably develops in a relatively short time frame and CRPC thus remains a uniformly fatal disease.

Clonal populations of cancer cells are in persistent evolution in response to environmental conditions. The concept of tumor evolution was proposed by Nowell in 1976 based on cytogenetic data (Nowell 1976). In this early model, a cell of origin acquires genetic alterations that promote neoplasia. Further genetic instability fuels clonal expansion of "fit" clones that ultimately leads to advanced malignancy, metastasis, and emergence of therapy resistance (Nowell 1976). Large-scale genome sequencing studies in recent years have provided expansive catalogs of the genomic aberrations in primary and advanced prostate cancers and have offered insight into deregulated molecular pathways at each stage of disease for the development of novel targeted therapies (Abeshouse *et al.* 2015, Robinson *et al.* 2015). However, these large-scale genome sequencing studies have focused on single samples of bulk tumors collected at a single time point from individual patients, which does not enable a complete understanding of tumor heterogeneity. An additional limitation with this single-site/single-time sampling approach is that it does not reveal the subclonal changes that occur within tumor populations spanning disease progression and recurrence after therapeutic intervention.

More recently, small-scale studies focused on sampling of multiple metastatic sites or longitudinal sample collection combined with computational reconstructions of clonal evolution have confirmed that primary tumors and adjacent normal tissue consist of multiple clonal populations. These studies have further revealed that metastatic spread can occur through monoclonal or polyclonal seeding between metastases or in waves originating from the primary tumor. Moreover, genomic alterations associated with resistance to ADT have been identified, including AR and AR pathway components (further expanded upon and reviewed in Watson *et al.* (2015)), highlighting an opportunity for the development of biomarkers representative of resistant subclones *vis-a-vis* personalized medicine approaches. In this review, we discuss the recent efforts to understand clonal evolution and map the clonal spread and expansion of metastatic prostate cancer. Collectively, these studies have illuminated several intricate mechanisms by which discrete clones undergo selection and spread in individual patients.

Genomic approaches to interrogate clonal framework in tumor tissues

The multifocal and heterogeneous nature of prostate cancer can hinder efforts to understand tumor cell clonality, particularly in metastatic disease. Until recently, the tools available to assess clonality within multifocal and heterogeneous tumor samples relied upon histological assessment, cytogenetic approaches such as FISH, and molecular approaches such as PCR. Many malignancies exhibit multiclonality including AML, breast, melanoma, esophageal, and non-small-cell lung cancer (Merlo *et al.* 2010, Ding *et al.* 2012, Nik-Zainal *et al.* 2012, Bolli *et al.* 2014, McFadden *et al.* 2014, Rashid *et al.* 2014). By applying the concept of population genetics of the most recent common ancestor, it has been possible to quantify genomic aberrations and thereby define clonal and subclonal populations of cells responsible for metastatic seeding and evasion of therapy (Campbell *et al.* 2008, Nik-Zainal *et al.* 2012). The first step in this strategy is to determine tumor purity, which is critical because resected tumors and biopsied tumor tissues harbor stromal cell infiltrates. This can be accomplished by identifying the fraction of tumor cells carrying clusters of mutations relative to normal tissue. (Van Loo *et al.* 2010). To define clonal and subclonal populations of cells within metastases at different body sites, mutant allelic fraction in multiple tumor sections relative to normal tissues can be calculated, taking into account tumor purity and copy number (Campbell *et al.* 2008, Nik-Zainal *et al.* 2012). Any mutations present at a smaller proportion in comparison with the clonal population would be indicative of subclonal events.

Using these methods, clonal evolution can be tracked across various disease states for which there is tissue available and can be used to reconstruct a spatiotemporal mutational landscape of disease progression. For example, this approach was used to retrospectively construct a time line of clonal evolution and metastatic spread in a rapid autopsy study of ten subjects that died from prostate cancer (Gundem *et al.* 2015). Alternatively, these methods enable monitoring of clonal evolution in real time. For example, one study collected longitudinal samples of blood and tumor biopsies from four patients with advanced prostate cancer as their disease progressed from localized prostate cancer, to biochemical recurrence, and ultimately to CRPC (Hong *et al.* 2015). A separate study examined clonal evolution in response to selective pressures of therapy by targeted sequencing of plasma DNA and targeted deep sequencing of tumor biopsies obtained from patients

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with ERG-positive cancers (Carreira *et al.* 2014). Together, these studies build upon an existing catalog of known mutations, structural alterations, and altered pathways to inform our understanding of the clonal evolution and spread of prostate cancer, particularly in response to AR-targeted therapies.

Primary prostate cancer is multifocal and heterogeneous

Prostate cancer often presents as discrete foci within the prostate capsule (Ruijter *et al.* 1999). Whether these discrete foci represent independent clones or geographically separated, yet related, clonal populations has been a subject of debate (Cheng *et al.* 1998, Barry *et al.* 2007, Kobayashi *et al.* 2008, Boyd *et al.* 2012, Lindberg *et al.* 2013, Boutros *et al.* 2015, Cooper *et al.* 2015). For example, in a study of 254 prostatectomy specimens, nearly half harbored multiple individual tumor foci separated by at least 3 mm within the resected gland (Villers *et al.* 1992). In these cases, it is possible that two distinct tumor cell populations arose in the prostate. Alternatively, independent foci may represent related clonal populations. Similarly, a study of 17 radical prostatectomy specimens indicated the presence of multiple tumor foci in several samples. Analysis of discrete foci revealed concordant as well as discordant allelic imbalances, suggesting variability in clonal origin of tumor foci within an individual prostate gland (Ruijter *et al.* 1999). In a separate study of 47 prostatectomy specimens, ~20% were multifocal and exhibited varying grades of disease. Loss of heterozygosity (LOH) was observed within tumors; however, intertumoral LOH was not predictable from intratumoral patterns of allelic loss (Hugel & Wernert 1999).

Primary prostate cancer can be subclassified according to a recurrent set of mutually exclusive genomic alterations that occur early in disease development (Abeshouse *et al.* 2015). Specifically, analysis of 333 primary prostate carcinomas in The Cancer Genome Atlas (TCGA) project identified seven mutually exclusive genetic subtypes including *ERG*, *ETV1*, *ETV4*, *FLI1*, *SPOP*, *FOXA1*, and *IDH1* (Abeshouse *et al.* 2015). Roughly 75% of prostate cancers fell into one of these genetic subtypes; however, ~25% remained uncharacterized (Fig. 1). Identification of additional genetic alterations has provided evidence that primary tumors exhibit multiclonality coincident

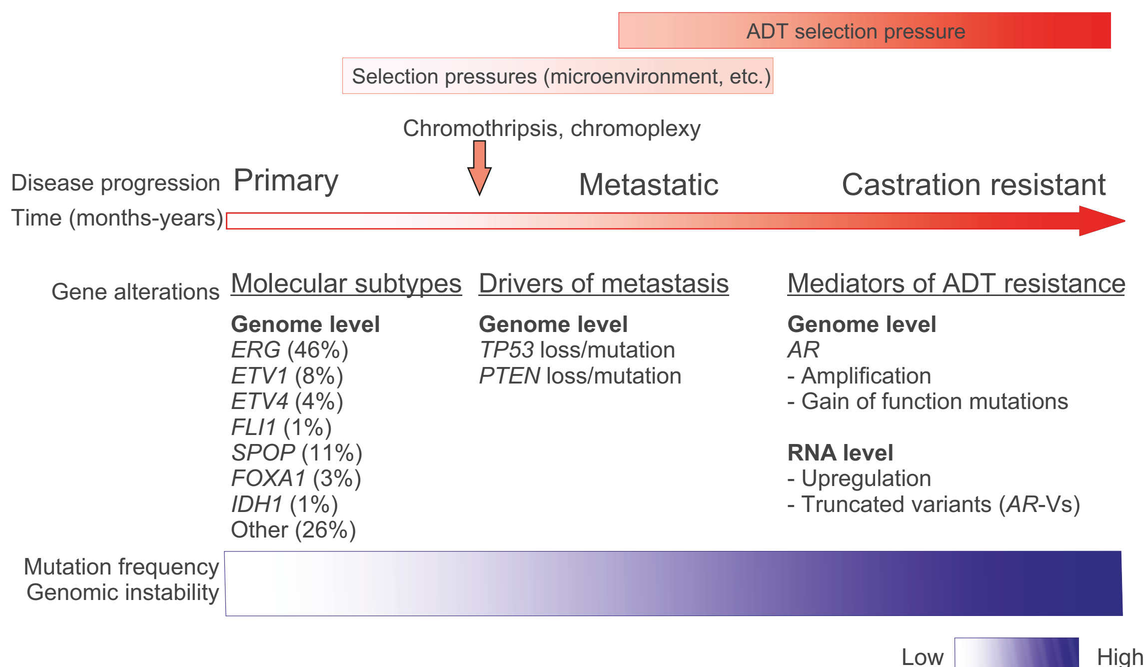

Figure 1
Schematic of common gene and pathway alterations across prostate cancer disease states, as identified by computational reconstructions of tumor evolution. Environmental factors impart selection pressures on clonal populations to drive metastasis and, ultimately, resistance to ADT. The TCGA study identified seven mutually exclusive genetic subtypes, listed here by percentage of tumors (333 total) (Abeshouse *et al.* 2015). *TP53* and *PTEN* inactivation are observed at higher frequencies in metastatic CRPC versus primary prostate cancer and are thought to play driver roles in metastasis (Abeshouse *et al.* 2015). Mediators of ADT resistance were observed to occur after metastatic spread (Carreira *et al.* 2014, Gundem *et al.* 2015, Hong *et al.* 2015, Romanel *et al.* 2015) and have been further reviewed in Watson *et al.* 2015.

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with multifocal disease. For example, whole-genome sequencing (WGS) in a cohort of five patients (Gleason scores 7–8) who underwent radical prostatectomies provided the first WGS study demonstrating extensive genomic heterogeneity within prostate tumors (Boutros *et al.* 2015). One example of multiclonality within the resected prostate was a patient whose tumor was dissected into four regions, each of which harbored disease foci. The index lesion shared *SPOP* mutations and chromosomal deletions (on chr8, 16) with one focus, but not the remaining two dissected foci, which shared a deletion on chr19 (Boutros *et al.* 2015). Importantly, this study also discovered a previously unidentified recurrent amplification of *MYCL* associated with *TP53* loss (Boutros *et al.* 2015). Similarly, morphologically normal tissues have been found to exhibit high levels of mutations and distinct *ERG* fusions that are present in malignant tissues (Cooper *et al.* 2015). In many cases, discrete tumor foci within the prostate comprised of distinct clones representing different genetic subtypes or harboring different driver mutations, indicating independent clonal origins. For example, interrogation of two independent foci present in a prostatectomy specimen demonstrated that they were distinct in clonal origin: one was ERG positive, whereas the other exhibited *SPOP* mutation (Barbieri *et al.* 2012). It is important to note, however, that in this cohort (112 patients), the authors reported only this single case of multifocal disease. In a smaller study of four patients, three discrete prostate cancer foci had no common somatic ancestor as assessed by high-frequency single-nucleotide variants (SNVs) and copy number alteration profiling (Lindberg *et al.* 2013). In a study of 48 patients with ERG-positive prostate cancer, areas of high-grade prostatic intraepithelial neoplasia (PIN) and intraductal carcinoma proximal to areas of invasive carcinoma were found to be ERG positive (Haffner *et al.* 2015). High-resolution studies of tissues from seven of those patients demonstrated that subclonal *PTEN* loss present in the ERG-positive invasive adenocarcinoma was also present in adjacent PIN lesions, indicating retrograde spreading to benign structures (Haffner *et al.* 2015).

Gradual acquisition of genomic alterations is considered to be the primary driver of tumorigenesis. However, accelerated mechanisms of genomic alteration, such as chromothripsis, a massive and catastrophic reshuffling of entire chromosomes or regions of chromosomes, and chromoplexy, complex rearrangements that may arise from multiple rounds of DNA repair, could account for shortened time frames of tumorigenesis and metastasis (Stephens *et al.* 2011, Baca *et al.* 2013). Indeed, chromoplexy has been shown to capture multiple genetic hits in one structural rearrangement event, which would be predicted to accelerate tumorigenesis and clonal evolution. For example, in one case of prostate cancer, the coding regions of several tumor suppressor genes, including *ETV6, ETV3,* and *CDKN1B,* were disrupted by a single chromoplexy event involving 25 rearrangements spanning three chromosomes (Baca *et al.* 2013). In another case of prostate cancer, 27 rearrangements occurred in a single chromoplexy event to yield an SMAD rearrangement as well as an oncogenic TMPRSS2:ERG fusion (Baca *et al.* 2013).

The most common lesions in primary tumors, affecting ~50% of cancers, are ETS fusions, in which AR-regulated or otherwise highly active promoters and/or enhancers such as *TMPRSS2* are fused to the coding regions of *ERG*, *ETV1*, *ETV4*, or *FLI1* (Taylor *et al.* 2010, Barbieri *et al.* 2013, Abeshouse *et al.* 2015, Adamo & Ladomery 2015). Interestingly, these gene fusion events have been shown to be promoted by androgen/AR-regulated changes in intra-nuclear chromatin organization (Lin *et al.* 2009, Mani *et al.* 2009, Weischenfeldt *et al.* 2013). For example, AR binding sites have been shown to exist near TMPRSS2, *ERG,* and *ETV1* fusion break points, and androgen-induced binding of AR to these sites has been shown to bring 5′ and 3′ gene fusion partners into close proximity (Lin *et al.* 2009). Genotoxic stress has been shown to cooperate with the androgen-mediated proximity effect to induce *TMPRSS2*:*ETS* or *TMPRSS2*:*ETV1* gene fusion events similar to those observed in clinical prostate cancer (Lin *et al.* 2009, Mani *et al.* 2009). Interestingly, this androgen-mediated proximity effect may promote the genesis of additional rearrangement events in the prostate cancer genome, as AR binding sites are frequently found adjacent to rearrangement break points (Weischenfeldt *et al.* 2013).

Clonal evolution and spread of metastatic prostate cancer

Relative to metastatic disease, primary tumors exhibit low mutation frequencies and genome instability (Abeshouse *et al.* 2015, Robinson *et al.* 2015). As the disease progresses to the metastatic and castration-resistant phases, extensive mutations, structural rearrangements, and genome instability are observed. These genomic alterations are indicative of clonal evolution and subclonal selection to survive environmental pressures. Importantly, altered AR signaling after metastatic spread spans subtypes occurs in more than 50% of metastases and remains a key target for therapies (Robinson *et al.* 2015). Commonly identified genomic alterations in metastatic CRPC are *TP53* mutation or loss,

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PTEN loss, PI3K pathway defects, DNA repair pathway deficiencies, and amplification or mutation of AR (Fig. 1) (Kim *et al.* 2007, Grasso *et al.* 2012, Lohr *et al.* 2014, Hong *et al.* 2015, Robinson *et al.* 2015). It has been challenging to differentiate alterations that promote metastasis vs alterations that promote therapy resistance because, historically, studies of metastatic prostate cancer have been performed using tissues from heavily treated CRPC patients. Exome sequencing of CRPC metastases obtained at rapid autopsy from a cohort of 50 patients identified frequent mutations in *TP53*, *AR*, *FOXA1*, *PTEN*, and among others (Grasso *et al.* 2012). Indeed, the finding that mutation or amplification of *AR* does not occur in primary prostate cancer but is a common genomic alteration in CRPC metastases appears to implicate AR as a driver of metastasis (Abeshouse *et al.* 2015). However, more detailed whole-genome sequencing studies have indicated AR as a driver of therapy resistance, rather than a driver of metastasis (Fig. 1) (Carreira *et al.* 2014, Gundem *et al.* 2015, Hong *et al.* 2015, Romanel *et al.* 2015). For instance, interrogation of subclonal structure within CRPC metastases demonstrated that different *AR* gene alterations can occur in different metastatic foci within a single patient, indicating that these alterations occurred in response to therapy, after metastatic spread (Gundem *et al.* 2015). However, this same analysis indicated that *TP53* loss, DNA repair pathway alterations, and *PTEN* loss occurred before metastatic spread (Gundem *et al.* 2015). Further, the frequencies of *TP53* and *PTEN* inactivation were higher in metastatic CRPC compared with primary prostate cancer, indicating driver roles in metastasis (Abeshouse *et al.* 2015).

Clonal spread between the primary tumor and distant metastatic sites is multidirectional

Metastases are considered to be monoclonal in origin with respect to the primary tumor, with clones sharing a number of common mutations but exhibiting divergent somatic mutations between different metastatic sites (Gundem *et al.* 2015, Hong *et al.* 2015). Early rapid autopsy investigations observed that PSA immunostaining varied across and within metastatic foci, suggesting that multiple subclonal populations existed within metastases (Roudier *et al.* 2003, Shah *et al.* 2004). Genome-wide copy number analysis and targeted sequencing studies revealed that metastases within a patient shared common mutations and therefore shared clonal origins (Liu *et al.* 2009, Robbins *et al.* 2011). However, these same studies also demonstrated that these tumors harbored sets of divergent mutations, indicating that subclonality existed between individual tumor foci. For example, analysis of three spatially distinct metastases from a single autopsy subject revealed shared amplifications at chromosomes 5p and 14q as well as a set of shared somatic mutations (Robbins *et al.* 2011). The presence of common copy number alterations and somatic mutations across multiple metastases indicated a clonal progenitor cell, presumably from the primary tumor. However, in this study, primary tumor tissue was not available for this patient (Robbins *et al.* 2011). Similarly, copy number analysis and targeted resequencing of 80 anatomically distinct metastatic foci isolated from 24 patients with CRPC indicated that metastases shared a clonal origin in most patients (Liu *et al.* 2009). Importantly, metastases shared clonal copy number variations with the primary tumor in five patients with available tissues (Liu *et al.* 2009). These observations indicate that prostate cancer metastases originate from a common clonal progenitor in the prostate. However, as discussed below, alternate explanations may also be possible in light of recent data, demonstrating that cells from prostate cancer metastases may be able to reseed the surgical bed where the original primary tumor existed (Liu *et al.* 2009, Hong *et al.* 2015).

A commonly accepted model of metastasis depicts subclonal populations colonizing metastatic sites in waves originating from the primary tumor (Wan *et al.* 2013). In this model, subclonal populations within the primary tumor compete for dominance and are selected for survival and growth by environmental selection pressures (Greaves & Maley 2012). In support of this model, a longitudinal study of four patients demonstrated that metastases were seeded in temporally separated waves originating from the primary tumor (Hong *et al.* 2015). Presumably, as the primary tumor acquired new mutations, structural variations, and copy number alterations, new metastatic subclonal populations were released to seed and reseed organ sites (Hong *et al.* 2015) (Fig. 2A). Similarly, in a single case study, anatomically distinct metastatic sites from a patient with CRPC were found to share many genetic alterations including high-level amplification of the *AR* locus, *PTEN* loss, *TP53* loss, and mutation of *SPOP*. As a result of these shared genomic alterations, these clones were interpreted to be monoclonal in origin (Haffner *et al.* 2013). Interestingly, examination of the microdissected primary tumor revealed that a small 2.2×1.3 mm well-differentiated Gleason pattern 3 lesion exhibited PTEN-negative immunohistological staining. DNA sequencing of this lesion revealed the same 4 base pair deletion in *PTEN* observed in the metastatic clones concurrent with *TP53* mutation, suggesting that this lesion harbored the

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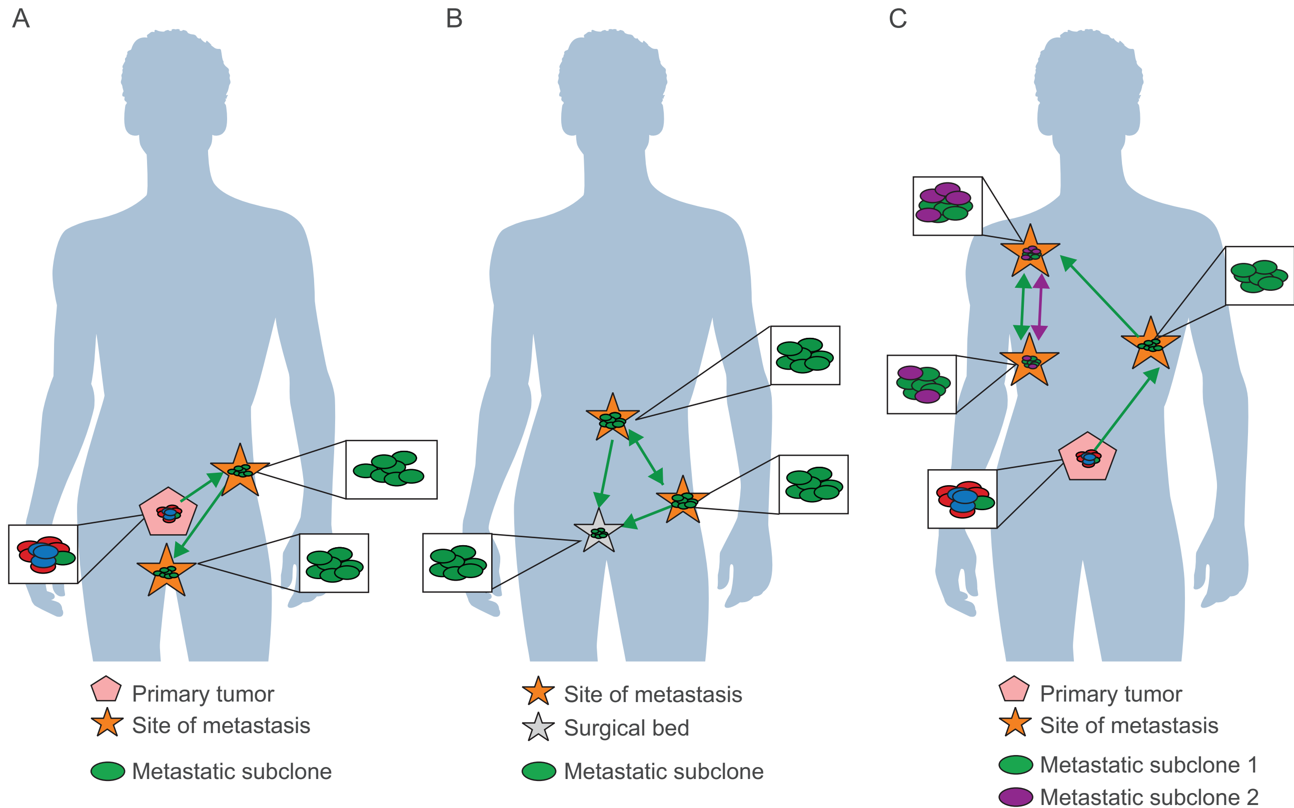

Figure 2
(A) A rare clone (green, "metastatic subclone") within the primary tumor acquires metastatic potential and seeds distinct metastatic sites in temporal waves. Clonal populations originating in the primary tumor can seed sequential anatomic sites such as the pubis and penis in a temporally distinct fashion (depicted by green arrows) (Hong *et al.* 2015). (B) Metastatic clones (green, "metastatic subclone") that seeded multiple sites (depicted by green arrows), including the sacrum and iliac crest in a bidirectional manner, can also reseed the primary surgical bed (Hong *et al.* 2015). (C) Clonal populations from metastatic sites seed anatomically distinct sites of metastasis in a polyclonal fashion. Here, a hypothetical metastatic clonal population (green, "metastatic subclone") seeds the left rib and shoulder (depicted by green arrows). A subclonal population arises in the shoulder (purple, "metastatic subclone 2"), and together, these populations colonize the rib (adapted from Gundem *et al.* 2015).

progenitor cell that seeded distant metastases (Haffner *et al.* 2013). Surprisingly, the surrounding higher grade Gleason pattern 4 tumor tissue did not harbor the same underlying mutations as the metastases. These data indicate that the primary index lesion does not always harbor the lethal clone that is ultimately responsible for seeding distant metastasis in the patient.

Another common mechanism of metastasis in advanced prostate cancer is via seeding of de novo metastatic sites by cells originating from distant metastasis (Gundem *et al.* 2015) (Fig. 2B). Furthermore, "local recurrences" after surgery may also arise via a similar mechanism of cells from distant metastases seeding the prostatic cavity after radical prostatectomy (Hong *et al.* 2015) (Fig. 2B). The mechanistic underpinnings of metastasis-to-metastasis and metastasis-to-primary site seeding remain to be clarified.

Multidirectional spread can lead to polyclonal seeding of metastatic sites

Similar to primary prostate cancer, a common model to explain intratumoral heterogeneity at metastatic foci has been that cells acquiring new genetic alterations are under constant environmental and therapeutic selection pressure, leading to continuous expansion and contraction of tumor subclones. However, in addition to this monoclonal model of metastatic evolution and spread, a polyclonal pattern has also been described. For example, Gundem and colleagues assessed the clonal relationship between subclones occupying different metastatic sites. On a phylogenetic tree, a truncal mutation in a pair of samples would be apparent if the same mutation was present in 100% in the cancer cell fraction at two different metastatic sites. Conversely, non-truncal (or branch) mutations would be apparent if the same mutation was present in less than

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100% of the cancer cell fraction at two different metastatic sites. Using this logic, it was found that 50% of the subjects in a rapid autopsy study exhibited polyclonal seeding of multiple metastatic sites (Gundem *et al.* 2015). Polyclonal seeding was defined in this study as multiple genetically distinct subclones colonizing a single metastatic site (Fig. 2C). Interestingly, all instances of polyclonal seeding involved mutations in genes with known involvement in therapy resistance, indicating that polyclonal seeding and interclonal cooperation may be required to evade therapy.

Overall, these studies have illuminated details of the complex mechanisms underlying metastatic seeding. This process can be initiated by monoclonal or polyclonal populations originating in both the primary tumor and metastases in other sites (Gundem *et al.* 2015, Hong *et al.* 2015). Polyclonal populations involved in these seeding events were more similar to one another than to clones occupying other metastatic sites (Gundem *et al.* 2015). Whether that is due to proximity effects on subclonal evolution or, conversely, characteristics of the metastatic niche that impart habitability remains to be tested. Importantly, it has become clear from these collective data that multiple mechanisms of seeding give rise to metastases in different body sites, highlighting the need for personalized medicine *via* monitoring of genetic changes in tumor cells within patients over the course of their disease and stages of treatment.

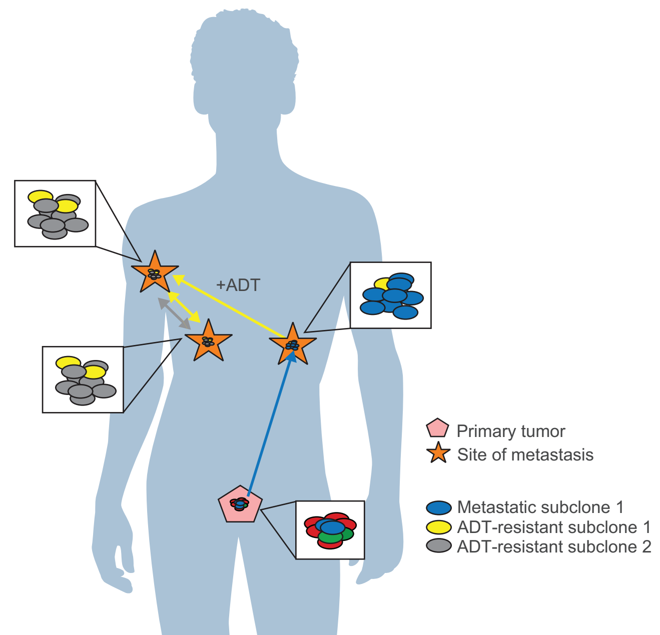

Figure 3
Treatment with hormone therapy selects and expands subclones harboring *AR* amplification and mutations to confer treatment resistance. A rare tumor clone (blue, "metastatic subclone 1") acquires metastatic potential and seeds the falciform ligament (depicted by a blue arrow), where subclonal populations acquire new mutations. Upon treatment with ADT, a subclonal population harboring mutations that promote ADT resistance (yellow, "ADT-resistant subclone 1") undergoes clonal expansion and spreads to a site in the axillary lymph node (depicted by yellow arrows). Acquisition of new mutations also occurs to generate another subclonal population resistant to ADT (gray). The mixture of ADT-resistant clones seeds the R. rib in a polyclonal manner (depicted by yellow and gray arrows, adapted from Gundem *et al.* 2015).

Subclonal expansion to escape targeted therapy

Treatment with AR-targeted ADT imparts a selection pressure upon tumor foci (Fig. 3). Clones harboring gene alterations that promote ADT resistance, such as *AR* point mutations, alterations in AR pathway components, and alterations in *MYC* and *CTNNB1*, were shown to seed and reseed multiple sites (Gundem *et al.* 2015). Those rare subpopulations of cells within tumor foci that reactivate AR through acquisition of mutations, copy number alterations, or synthesis of constitutively active AR splice variants will evade ADT (Watson *et al.* 2015). This concept is supported by a recent targeted sequencing study in which germ line, plasma, and tumor samples from ERG-positive patients were collected before, during, and after treatment with abiraterone acetate or enzalutamide. This longitudinal analysis identified AR amplification and the appearance of AR point mutations in response to therapy (Carreira *et al.* 2014). An additional mechanism of increased AR signaling in response to ADT is expression of truncated AR variants (Dehm *et al.* 2008). Expression of AR-Vs has been detected in primary tumors, metastases, as well as circulating tumor cells (Abeshouse *et al.* 2015). AR-Vs function as constitutively active transcription factors and can enable CRPC cells to escape AR-directed therapies that require the intact AR ligand binding domain (Dehm *et al.* 2008, Guo *et al.* 2009, Sun *et al.* 2010, Watson *et al.* 2010, Li *et al.* 2011, Hu *et al.* 2012). Intriguingly, RNA-seq studies have indicated that AR-V expression relative to full-length AR does not increase dramatically in metastatic CRPC tissues relative to primary tumor tissues (Abeshouse *et al.* 2015, Robinson *et al.* 2015). This finding suggests that subclonal expansion of AR-V-expressing cells does not occur in metastatic disease and may instead represent a property of rare populations of tumor cells that are resistant to ADT. However, these findings from RNA-seq-based studies are in contrast to RT-PCR-based studies, which have found higher expression of AR-Vs in CRPC tissues relative to primary prostate cancer, as well as correlations between AR-V expression and with poor outcomes (Hu *et al.* 2009, Sun *et al.* 2010, Hornberg *et al.* 2011).

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One provocative observation in recent studies has been that the acquisition of various *AR* mutation and amplification events at metastatic sites can occur through polyclonal seeding mechanisms (Fig. 3) (Gundem *et al.* 2015, Hong *et al.* 2015). For example, in one patient, two subclones involved in polyclonal seeding harbored two different alterations that promote ADT resistance, including *MYC* amplification and a pathogenic AR substitution (Gundem *et al.* 2015). Similarly, in a longitudinal biopsy study, a metastatic site in the iliac crest displayed profound clonal evolution in response to ADT, ultimately becoming enriched for a subpopulation of cells that had originated in a sacral metastasis (Hong *et al.* 2015). In patients that exhibited polyclonal seeding, several subclones across metastases were shared and were therefore more similar to each other than to the primary tumor (Gundem *et al.* 2015). Together, these findings suggest that interclonal cooperation may occur and that multiple independent ADT-resistant subclones may arise and ultimately cooperate to drive subclonal expansion in response to ADT. There is precedent for polyclonal seeding in murine models of mammary tumors; however, until recently, this has not been observed in human tissues (Cleary *et al.* 2014, Marusyk *et al.* 2014). For example, in WNT-driven mammary tumors, $HRAS^{mt}$ WNT^{low} basal and $HRAS^{wt}$ WNT^{high} luminal subclones cooperate interclonally to propagate tumor growth and response to WNT-targeted therapy (Cleary *et al.* 2014). In these cases, acquisition of multiple mutations across subclonal populations appears to promote and may be necessary to drive tumor growth and clonal expansion under treatment conditions.

Clones representative of multiple stages of disease persist in blood

Tumor cells representing various stages and sites of disease are detectable in blood. Thus, circulating tumor cells and cell-free circulating tumor DNA provide a window into the totality of disease within a patient. Recently, a number of studies have demonstrated detection of biomarkers of advanced disease and therapy resistance in patients with advanced prostate cancer. For example, a clone originating from a primary tumor was detected by targeted sequencing of genomic DNA isolated from blood 3 years after resection of the prostate (Hong *et al.* 2015). In the same patient, a metastatic clone that originated in the primary tumor but seeded a metastatic site in the seminal vesicle was detectable in blood (Hong *et al.* 2015). Targeted sequencing of DNA isolated from plasma revealed the emergence of T878A and L702H mutations in the *AR* during progression on abiraterone (Romanel *et al.* 2015). In the same study, patients with AR amplification or *AR* mutation were less likely to experience a PSA decline after initiation of therapy with abiraterone acetate. Importantly, the presence of mutant AR alleles was more common in patients that did not display evidence for *AR* gene amplification, indicating that clones exhibiting single alterations in *AR* may be sufficient to impart therapy resistance (Romanel *et al.* 2015). Accordingly, *AR* amplification was detected in circulating cell-free DNA and was shown to be associated with enzalutamide and abiraterone treatment resistance in a cohort of 62 CRPC patients, albeit at a higher frequency in those treated with enzalutamide versus those treated with abiraterone acetate (Azad *et al.* 2015).

AR variants that are detectable at the mRNA level in circulating tumor cells are predictive of poor response to treatment with abiraterone acetate or enzalutamide (Antonarakis *et al.* 2014, 2015). In a cohort of patients with castration-resistant disease, more than 50% of patients had detectable expression of at least one AR-V mRNA in single circulating tumor cells isolated from blood, and ~17% expressed more than one AR-V in an individual circulating tumor cell (Miyamoto *et al.* 2015). In the same study, the authors observed alterations in GR and Wnt signaling in different subsets of circulating tumor cells. Variations in subclonal abundance were observed in patients with CRPC before, during, and after treatment with abiraterone (Carreira *et al.* 2014). In one patient, biopsy of a liver metastasis revealed a W742C mutation in *AR*, whereas circulating DNA revealed *AR* amplification. Treatment with docetaxel resolved the liver metastasis; however, the patient exhibited no response to abiraterone acetate, as defined by a 50% decline from baseline in serum PSA levels. This was likely due to the occult subclonal lesions harboring *AR* amplification, which were detectable in plasma DNA (Carreira *et al.* 2014). Together, these studies indicate the presence of multiple subclonal populations within the circulation of a single patient. Overall, capture of circulating tumor cells or cell-free DNA has provided a useful approach to gain a snapshot of subclonal populations and their evolution during disease progression. As such, these studies have generated enthusiasm around the ultimate use of circulating tumor cell or cell-free DNA analysis to help inform treatment decisions for individual patients.

Summary

Retrospective and real-time computational renderings of the clonal architecture within patient primary tumors and

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metastases have provided an unprecedented window into clonal evolution across progressive disease. A tremendous amount of genetic information is available describing the mutations, structural alterations, and altered pathways in both primary and metastatic prostate cancer, yet they are limited in that they can only provide genetic alterations across bulk tissue samples (Grasso *et al*. 2012, Abeshouse *et al*. 2015, Robinson *et al*. 2015). Here, we have reviewed studies that confirm the clonal heterogeneity of primary and metastatic prostate cancer and provide early evidence that metastatic spread can occur via several mechanisms and not one distinct linear pathway. Furthermore, the studies discussed here have provided the first insights into the temporal acquisition of gene alterations during metastasis and therapy resistance of prostate cancer.

A consensus finding from these studies is that loss or mutation of *TP53* and *PTEN* loss occur before or early in metastasis, indicating that they drive metastatic spread. The recently identified mutually exclusive molecular subtypes of primary prostate cancer including *ETS* fusions and mutations in *FOXA1, FLI1, SPOP,* and *IDH1* represent early drivers of tumorigenesis, and at least some molecular basis for inter-patient heterogeneity. However, acquisition of mutations in drivers of metastasis does not appear to occur preferentially in any one of these molecular subtypes, indicating that the transition to metastatic disease may follow a somewhat unified biological mechanism. Another key finding in these studies is that AR, which is altered in >60% of metastatic prostate cancer and known to be a mediator of ADT resistance (reviewed in Watson *et al*. 2015), undergoes alterations after metastasis has occurred. This novel finding indicates that metastasis and therapy resistance are temporally separate processes. However, it remains unclear whether rare subclones originating in the primary tumor or early metastases harbor AR alterations that later promote ADT resistance or, alternatively, whether such alterations arise after metastasis and initial treatment with ADT. Further analysis of circulating tumor cells from patients at various stages of disease progression may help to understand whether resistant tumor clones are present before ADT.

In addition to providing insight into the temporal landscape of genetic alterations across prostate cancer development, the studies reviewed here have provided views of the mechanisms of metastatic spread. Foremost, these studies have established that metastatic spread can occur through monoclonal or polyclonal seeding between metastases or in waves originating from the primary tumor. Furthermore, they have demonstrated that clonal spread is not unidirectional, as metastatic clones can reseed the surgical bed of the resected prostate. The mechanistic underpinnings of metastasis-to-metastasis seeding remain to be understood. For example, the properties of the metastatic niche that are required for either monoclonal or polyclonal seeding or reseeding by metastatic clonal populations are largely unknown. Nevertheless, these studies have highlighted the complexity of patterns of clonal spread and subclonal evolution in response to environmental and therapeutic pressures such as ADT. Monitoring circulating tumor cells and circulating cell-free DNA provides an opportunity to track the totality of these complex disease processes within an individual patient and possibly identifies subclones harboring clinically actionable mutations or early indicators of therapeutic resistance.

Declaration of interest

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