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The Cohesive Metastasis Phenotype in Human Prostate Cancer

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Abstract

A critical barrier for the successful prevention and treatment of recurrent prostate cancer is detection and eradication of metastatic and therapy-resistant disease. Despite the fall in diagnoses and mortality, the reported incidence of metastatic disease has increased 72% since 2004. Prostate cancer arises in cohesive groups as intraepithelial neoplasia, migrates through muscle and leaves the gland via perineural invasion for hematogenous dissemination. Current technological advances have shown cohesive-clusters of tumor (also known as microemboli) within the circulation. Circulating tumor cell (CTC) profiles are indicative of disseminated prostate cancer, and disseminated tumor cells (DTC) are found in cohesive-clusters, a phenotypic characteristic of both radiation- and drug-resistant tumors. Recent reports in cell biology and informatics, coupled with mass spectrometry, indicate that the integrin adhesome network provides an explanation for the biophysical ability of cohesive-clusters of tumor cells to invade thorough muscle and nerve microenvironments while maintaining adhesion-dependent therapeutic resistance. Targeting cohesive-clusters takes advantage of the known ability of extracellular matrix (ECM) adhesion to promote tumor cell survival and represents an approach that has the potential to avoid the progression to drug- and radiotherapy-resistance. In the following review we will examine the evidence for development and dissemination of cohesive-clusters in metastatic prostate cancer.

Keywords

prostate cancer; cohesive-clusters; metastasis; integrins; circulating tumor cells; adhesome

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The Transparency document associated with this article can be found, in online version.

1. Introduction

The National Cancer Institute estimates 180,890 new cases of prostate cancer in 2016, with 26,120 estimated deaths [1]. Confined and localized prostate cancer generally is considered curable, while invasion beyond the prostate capsule, leading to metastasis, is associated with poorer prognosis and higher mortality. Between 1992 and 2013, there was a marked decrease in overall rate of diagnoses (from 234.2 to 104.6 per 100,000) and deaths (from 39.2 to 19.2 per 100,000) [1]. Yet, a recent report showed that the incidence of metastatic disease in the United States increased 72% between 2004 and 2013 in a sample of 767,550 men diagnosed with prostate cancer (from 1685 cases in 2004 to 2890 in 2013) [2]. Among the possible explanations for the significant rise in metastatic disease are changes in screening approaches, adaptations in the biological aggressiveness of prostate cancer, or increases in the discovery of metastatic disease. The latter option seems unlikely given that increased or better imaging would identify metastases in more men with lower prostate-specific antigen (PSA) scores, yet researchers found the opposite, an increase in PSAs among men with metastatic prostate cancer during this period [2].

Metastasis from the primary tumor to a distant organ is responsible for 90% of all cancer deaths [3,4]. During the last 10 to 15 years, research has increased steadily toward the goal of developing circulating tumor cells (CTCs) as minimally invasive biomarkers in cancer diagnosis and management. The detection, capture, and identification of CTC's in peripheral blood, a technique known as liquid biopsy, continues to be promoted as an alternative to surgical biopsies [5], and can be performed repeatedly with low risk for side effects. The only FDA-approved CTC collection technology, CellSearch, is based on detection of CTCs expressing the epithelial cell adhesion molecule (EpCAM), but it can only identify single CTCs and lacks the technology necessary to preserve CTC-cluster integrity or to reliably sort them [6,7]. However, new technology is reported that allows label-free isolation of unfixed CTC-clusters from unprocessed whole blood samples from patients with cancer [6,8]. In this review, we will examine the biology of cohesive CTC-clusters escaping the primary tumor and the survival advantage of clusters moving through the vascular system to seed a distant site as an alternate explanation for the success of metastatic prostate cancer.

2. Methods

In constructing this review, we used the most recent research available on the cohesive-cluster phenotype, with an emphasis on epithelial cancers. Contributions published from 2012 onwards primarily were used that were specific to the cohesive-cluster model of circulating tumor cells relevant to prostate cancer metastasis. In presenting more basic research, we chose to cite canonical studies when possible, especially when discussing general biologic structures or functions.

Review articles are cited when possible to balance the need for completeness and the citation of the most recent work in the area while working with a 100 citation limit. Since reviews also cite previous reviews, the ideas presented are several steps removed from the original data and may, unintentionally or not, represent the biases or cognitive filters of the prior

reviewers. We made every effort to ensure the ideas and data presented are as felicitous to the original research as possible.

3. Results

3.1. Prostate biology, cancer, local invasion, and metastasis

The human prostate is a complex tubuloalveolar gland with regions defined by concentric zones, including the anterior fibromuscular compartment, the central zone, the peripheral zone, and the transition zone. Prostate cancer arises specifically in the peripheral zone of the prostate gland and is distinct from benign prostatic hypertrophy (BPH) that arises most frequently in the transition zone [9]. The prostate gland is completely surrounded by a smooth muscle casing known as the prostate capsule, and a majority of epithelial tumors exhibit traits of collective invasion into surrounding tissues, including cell-cell adhesions, the presence of E-cadherin (and other cadherins), and occurrence of other cell-cell adhesion receptors in tumor areas within normal stroma [10]. The smooth muscle stroma of the human prostate gland is permeated by the cavernous nerve and neurovascular formations of the pelvic plexus that are comprised of autonomic nerves (reviewed in [11]).

Research has found that innervation of the prostate peripheral zone is considerably greater than that of the transition zone; accordingly, the greatest innervation was found in neurovascular bundles and seminal vesicles of the prostate's peripheral zone (reviewed in [12]). Significant innervation in the peripheral zone led to the notion that prostate tumors move along the nerves as a non-random event [12]. Tumor-cell groups in the peripheral zone appear to escape the prostate capsule, as a major progression in the disease, through invasion of prostatic nerves and neurovascular bundles in a process known as perineural invasion (PNI) (reviewed in [13]).

As shown in Fig. 1, cohesive groups of prostate cancer surround the nerve (perineural invasion) or invade into nerves (endoneural invasion). In support of this premise, studies have shown that approximately 85% of prostate cancer cases demonstrate PNI, as cell clusters escape along the cavernosal nerve, prostatic plexus, and neurovascular bundles [13]. A laminin adhesion receptor, α6β1 integrin, which is crucial to peripheral nerve development, is also used by prostate tumor-cells for migration, perineural invasion, and eventual metastasis to bone [13].

Prostate cancer is a neurotropic cancer (as are pancreatic, head and neck, and colorectal cancers) with a remarkable ability to appropriate the complex neural structures of highly-innervated organs as a means for primary tumor cell escape [14]. Our group has demonstrated that metastasizing prostate tumor cell-clusters invade along nerves (Fig. 1) containing and enabled by Schwann cells [13]. While the dominant view of epithelial cancer invasion holds that single tumor cells invade the surrounding stroma, preceding intravasation and dissemination [11], the weight of evidence suggests that prostate tumors are cohesive-clusters using perineural invasion [11,13–15].

The innermost layer of peripheral nerves, the endoneurium, contains myelin-forming Schwann cells [14] and, as seen in Fig. 1, the peripheral nerves are surrounded by a basal

lamina and a fibrillary reticular lamina that, in concert with surrounding collagen fibrils, comprise the endoneurium. A group working with pancreatic cancer found that Schwann cells guide cancer cells toward nerves and promote contact-based invasion, leading to the formation of cancer cell protrusions that generate cancer cell dissemination—and, importantly, they found that paracrine signaling and remodeling of the ECM were insufficient to trigger invasion in pancreatic tumors [15]. PNI occurs in 50–100% of pancreatic cancers and in 85% of prostate cancers—pancreatic tumor cells invade the surrounding parenchyma and penetrate the celiac plexus, whereas prostate tumor cells escape along the cavernosal nerve, prostatic plexus, and neurovascular bundles [14] leading to hematogenous dissemination. New technological advances [3,8] have led to the detection of the micro-metastases in model systems.

Recent work reveals that invasive cancer clusters can be directed by biomechanical cues in the tissue microenvironment (reviewed in [16]). Muscle is recognized as a structured and stiff tissue, compared to endothelial layers or adipose, and tumor clusters invading through muscle would be expected to acquire correspondingly different physical features, either by selection or in response to the biophysical constraints and dynamic tensile forces of the tissue [17]. Knowing that human prostate tumors invade and migrate as groups within a fibromuscular microenvironment and escape the gland aided by nerves and neurovascular bundles suggests the importance of further understanding the biophysical cues that promote cohesive-clusters in preventing metastatic spread. Invasive prostate cancers express α6β1 and α3β1 laminin-binding integrins, as well as laminin 511, the same laminin form that predominates within muscle and nerve microenvironments encountered by human prostate tumors (Fig. 1 and reviewed in [11]). These microenvironments provide the primary sources of paracrine signaling and biophysical cues to promote prostate cancer early invasion and metastatic events [14].

Identification of unique prognostic indicators and/or novel molecular targets directly involved in metastasis along nerve routes could invite an extraordinary change in how the disease is treated, with the potential to advance nerve-sparing radical prostatectomy techniques. For example, the peripheral zone has different motor neurons [13] than the anterior fibromuscular compartment, the central zone, or the transition zone of the prostate gland. Knowing which nerves the tumor uses in PNI might aid in developing precision nerve-sparing surgery techniques, alternative precision ablation methods (like focused ultrasound [18] or targeted and volumetric hyperthermia [19]), or specialized intraoperative imaging techniques to minimize the loss of associated motor nerves that lead to incontinence and impotence.

3.2. The cohesive-cluster phenotype in prostate cancer

While prostate cancer is associated with a favorable prognosis [20], there remains no definitive molecular marker to predict the subset of invasive disease that will disseminate. Prostate cancer metastasizes early to pelvic lymph nodes and, as distinct from other epithelial cancers, predominantly metastasizes to bone [21–23]. Metastases in the lymph node, vessels and bone are observed as clusters (Fig. 2). A treatment challenge is the knowledge that cancer cells escape early in disease progression (prior to surgery or

radiation) and can remain dormant in bone marrow for years before switching to a proliferative phenotype and triggering metastases development [23]. There currently is no way to detect these disseminated tumor cells (DTCs) as this process often occurs before treatment begins and the technology to detect DTCs is lacking.

Cohesive-clusters of human prostate cancer cells frequently are observed at multiple stages of dissemination (Fig. 2). Cohesive-clusters can be detected as an obturator lymph node metastasis [24], within the vasculature as clustered CTCs, or within bone (Fig. 2). When these cohesive-clusters have been studied, they contain no mitotic figures and are Ki-67-negative, both in breast cancer CTCs [25] and in prostate cancer metastases [21,23]. These findings suggest that single CTCs may not be the primary origin of metastatic tumors but, rather, that cohesive CTC-clusters, which have been identified as more efficient than individual CTCs in seeding distant metastases [26,27] (reviewed in [3]), should be a primary therapeutic target. However, more research is needed in order to understand how these clusters develop and function, as there is still much to be learned about the escape of tumor clusters from the primary tumor, movement through the vasculature, and dissemination to bone marrow. Determining the incidence of circulating clusters in patients with advanced disease versus patients with early stage disease would be an important area of study.

While “micro-metastases” are difficult to detect, cohesive CTC-clusters were often dismissed as an error when estimating CTC numbers, as conventional antibody-based CTC enumeration procedures only count single cells, and liquid biopsies appear unable to detect CTC-clusters [5]. More recent work using non-antibody-based approaches has shown that CTCs are distributed as cohesive-clusters as well as individual cells [7,10,27,28]. Friedl and Gilmour identify three basic properties of collective cell migration: (1) cell clusters remain connected and the cell-cell junctions are preserved; (2) multi-cellular polarity along with actin cytoskeleton organization produces adhesive friction and protrusion of the crawling edge of the cluster while preserving cell-cell junctions; and (3) clusters of moving cells often modify the tissue structure of vessels by which they travel, either by removing obstacles or by generating secondary modifications of the ECM, including the installation of a basement membrane [10].

An important aspect of CTC-cluster migration is the establishment of “leader” cells and “follower” cells within the cluster that have been found in all migrating collectives described —including morphogenesis, wound repair, and cancer invasion [10]. The molecular mechanisms underlying single-cell polarization and migration are well-known, and the same basic mechanisms are applicable in collective movement (reviewed in [29]). In single-cell migration, a front-to-rear polarity axis is generated, including polarized cytoskeletal reorganization and the polarized configuration of membrane trafficking. Rac and CDC42 instigate cytoskeletal reorganization of the front of the cell, including rapid actin polymerization—leading to the creation of membrane protrusions, such as filopodia and lamellipodia—and promote integrin engagement with the ECM; while at the cell's rear, the Rho signaling pathway triggers acto-myosin contraction [29]. In a recent study, researchers found that Rac, β1 integrin, and PI3K are upregulated in leader cells [30]. In collective migration, the same processes occur as do in single-cell migration; however, in cohesive-clusters cellular adhesions alter the distribution of functions found in isolated migrating cells

with those cells at the front of the cluster becoming leader cells, while those composing the remainder of the cluster becoming follower cells.

Leader cells are sensitive to the microenvironment and, therefore, control the direction and speed of migration of the cohesive-cluster, while being exposed to more external signals (for example, chemoattractants) and being largely responsible for ECM remodeling during migration. In the remainder of the cluster, cell-cell adhesions reduce formation of a classical leading-edge in the individual cells, suggesting that the mechanisms propelling the migration of follower cells are different from those influencing the leader cells [29]. Further, leader cells often appear less organized and mesenchyme-like, while cells at the rear of a cluster tend to demonstrate more adhesive assemblies, such as tubular networks, and contain tight cell-cell junctions generally absent in leader cells [10]. These differences in cellular processes in cohesive-clusters may offer an explanation for why the proposed epithelial-to-mesenchymal transition (EMT) has been difficult to prove (reviewed in [8,11,31]).

Microfluidic devices for label-free physical capture of the circulating cell clusters are being reported and, in one study using Cluster-Chip technology, cohesive tumor clusters were identified in 30–40% of patients with metastatic cancers of the prostate, breast, and melanoma [6]. Other reports demonstrate that clusters of up to 20 tumor cells can traverse capillary-sized vessels (5- to 10-μm) by quickly and reversibly transforming into a single-file, linked structure with considerably reduced hydrodynamic resistance and lower sheer forces [3]. In breast cancer, the number of CTCs detected in the bloodstream is significantly greater than the frequency of metastases found in patients, suggesting that the overwhelming majority of CTCs perish in the bloodstream, likely due to epithelial cells undergoing anoikis resulting from missing adhesion-dependent signals [26]. CTC-clusters, as distinct from tumor-cell aggregates, show a 23- to 50-fold increase in metastatic potential due, in part, to increased expression of cell-cell adhesion proteins within the clusters [26].

CTC-clusters contain tissue-derived macrophages, but do not contain other leukocyte subclasses, including T cells, B cells, hematopoietic stem cells (HSCs), or natural killer (NK) cells [6]. CTCs produce proinflammatory cytokines and chemokines that draw tumor associated macrophages (TAMs) to the tumor-cell clusters from circulation [32]. Macrophages are known to aid prostate cancer tumor-cell invasion and migration through modification of the adhesion function of laminin-binding integrins that interact with laminin 511 [32], suggesting that tumor clusters themselves contain a specialized pro-metastatic microenvironment. Increased retrieval of the CTCs, including the CTC-clusters and associated cells, will likely aid in treatment stratification of prostate cancer (reviewed in [5]). Taken together, these data suggest that cohesive-clusters of CTCs show much greater survival in circulation, are more likely to lead to metastasis, and may be a potent diagnostic marker for metastatic disease.

Recent findings demonstrate that evaluation of a single prostate cancer metastasis provides a reasonable assessment of the known oncogenic driver alterations that are present in intra-individual disseminated tumors [33], including the number of somatic mutations, genomic copy number alterations, measures of androgen receptor (AR) activity, and cell-cycle activity. New technological advances permit the retrieval of CTCs and CTC-clusters in

patients with metastatic castration-resistant prostate cancer (mCRPC) [3,6] and characterizing the molecular determinants may offer new strategies to prevent metastasis with early intervention. Others suggest that CTCs can be sampled to determine some aspects of the tumor biology, for example, AR expression and epidermal growth factor receptor (EGFR/Her1) overexpression [34], which makes the ability to analyze CTC-clusters potentially a powerful tool.

Notably, it has been found that AR activity was inversely associated with cell proliferation [33], supporting others' work showing that androgen depletion therapy (ADT) therapy increases tumor proliferation and dissemination [4,35]. There is increasing acceptance that mCRPC is not androgen-independent and continues to employ androgen signaling [4], despite systemic ADT [36]. Moreover, evidence suggests that mCRPC is an evolving entity, seemingly adapting to each additional therapy administered and adopting new and diverse resistance mechanisms, including reliance on androgen signaling despite therapeutic efforts to deplete all androgen production and disable receptors (reviewed in [36]). While AR inhibition with enzalutamide and abiraterone is initially successful in approximately 60 to 80% of patients with mCRPC, nearly all will develop secondary resistance [4, 35]. Gundem et al. comment that ADT inevitably leads to castration-resistant disease by several mechanisms, including: AR amplification, mutations that increase AR sensitivity [35], AR phosphorylation, and circumvention of the AR pathway [4]. The known recurrence and adaptations of mCRPC, along with the eventual ineffectiveness of ADT and subsequent treatments for mCRPC [33], indicate that strategies to prevent metastases are unmet clinical challenges [36].

Another study found that specific sites of metastasis are associated with overall survival time in men with mCRPC, with a shorter overall survival observed for lung and liver metastases as compared with bone and non-visceral involvement [37]. Yet, the lack of reliable serum markers that enable the identification of patients with mCRPC tumors transforming to untreatable neuroendocrine prostate cancer (NEPC) or equally lethal small-cell carcinoma (SCC) remains an important gap in our current knowledge. Taken together, these findings require a better understanding of variations in tumor phenotype, a greater comprehension of the biological determinants of different metastatic sites, and further investigation into the formation and dissemination of cohesive-clusters of tumor cells, all of which can inform treatment decisions and the design of future clinical trials [37].

3.3. Cohesive-cluster Phenotype: Single cells versus cohesive-clusters

The traditional model of tumor metastasis contends that single cells undergo epithelial-to-mesenchymal transition (EMT) within the primary tumor, leading to intravasation into the bloodstream, survival of single CTCs within the bloodstream, extravasation at a distant site, where mesenchymal-to-epithelial transition (MET) culminates in CTC proliferation as epithelial metastatic deposits [26], resulting in a clonal metastasis [38]. However, the evolutionary nature of cancer emergence leads to competing, genetically distinct clones that arise from single cells, while the different clones can occur within a single primary tumor [4,39]. Metastases can also be polyclonal, comprised of two or more genetically unique clones [27]. These observations coupled with the finding that human prostate carcinoma

primarily invades as a cohesive collective similar to embryonic processes [11] suggests that the cohesive-cluster phenotype is a common feature despite the genomic heterogeneity. The exception may be in the instance of rare single cell variants typical of Gleason Grade-5 tumors.

Importantly, cohesive-clusters of CTCs from a primary prostate tumor are reported to employ a partial EMT, adopting some mesenchymal characteristics but maintaining an "intermediate phenotype" that is epithelial in nature [5] (reviewed in [8]). As suggested above, this hybrid epithelial/mesenchymal (E/M) or partial-EMT phenotype could potentially allow CTC-clusters to exhibit a mixture of epithelial cell–cell adhesion (in the follower cells) and mesenchymal motility traits (in the leader cells), thereby supporting collective cell migration as seen in wound healing, tissue morphogenesis, and some cancer models [8].

Despite these findings, most of the published evidence supports the single-cell CTC model, based on Cell Search identification system, which detects EpCAM (epithelial cell adhesion molecule) antigen on the surface of CTCs [8]. This form of CTC isolation technology, as well as liquid biopsies [5], can only identify single CTCs and lacks specificity and neglects sample processing constraints required to maintain the integrity of CTC-clusters or to even sort them adequately [6]. There are several other options for capturing and analyzing CTCs [8], including the Cluster-Chip technology that uses the cell-cell junctions in CTC-clusters to isolate the clusters, with high sensitivity, from untreated blood samples [6].

Importantly, cell-cell cohesion as a phenotype of metastasis has been demonstrated in prostate cancer [Table 1], as well as in other epithelial cancers [3,8,15,26,40] (reviewed in [10,28]). One group, working on breast cancer with an *in vivo* mouse model, found that genetically unique tumor cells will form mixed clusters rather than simple clonal groups and single-cell injections of traceable tumor cells generated an average of zero to one metastasis per mouse, while aggregated clusters produced many large metastases with more than an 100-fold increase in metastatic efficiency compared to single cells [27]. A growing body of research suggests that cohesive-clustering significantly increases tumor cell survival as the cells move to distant sites and promote successful metastasis. Future work will likely determine if systemic approaches to inhibit cohesive clusters will prevent metastasis.

3.4. Cohesive metastasis phenotype aids therapeutic resistance and biophysical barriers of dissemination

Cell adhesion-mediated drug (CAM-DR) and radiation resistance (CAM-RR) represent major impediments to the successful treatment of cancer [38,42]. Epithelial-derived cancers, which are dependent upon cytokeratin [43–45] and integrin function (reviewed in [46]), are particularly resistant to the lethal effects of DNA-damaging agents, including most chemotherapeutic agents [44]. In epithelial tumors, targeting β1 integrin will significantly improve the therapeutic response to ionizing radiation [47]. In model systems, the 3D–growth of epithelial cancer cells mediates a significant increase in radiation- and chemo-resistance as compared to 2D–growth tissue culture conditions. The corresponding mechanism(s) are differential expression of genes involved in the regulation of integrin signaling, cell-cell contact [48], and enhanced cell-cycle progression blocks [31]. These

observations have stimulated the development of high-throughput screening technologies using 3D–growth conditions for discovery of agents that will act as sensitizers and possibly as adjuvants to chemotherapy and ionizing radiation treatments [49].

Although cohesive CTC-clusters can pass through very small, capillary-sized spaces [3]—a significant environmental stress—recent research has found that cells and cell-clusters passing through tight spaces (3 μm) are subject to much greater risk of nuclear envelope (NE) rupture and concomitant DNA damage [50]. However, it was also found that NE rupture in collective cell-clusters occurred less frequently than in individual cells, due to a tendency in clusters to migrate through low-resistance pathways, allowing the clusters to experience decreased levels of DNA damage. Further, during cancer cell migration, depletion of intermediate filaments that line the inner membrane of the nucleus can result in rupture of the NE and cause DNA damage that requires repair [50]. Increased DNA damage occurring during tumor cell migration would predict that those CTCs would have increased drug sensitivity. These observations may explain, in part, the increased sensitization of tumor cells to DNA-damaging agents that is dependent upon intermediate filament networks [43,44].

While the mechanisms involved in CAM-DR and CAM-RR are varied, current live-cell imaging has made it possible to appreciate the dynamic processes of intermediate filaments. Intact networks provide the structural integrity, including the “perinuclear cage,” to protect cells from environmental stresses and yet remain flexible and responsive to environmental cues (reviewed in [51]). Keratin 8 and 18 (K8/18) form intermediate filaments that surround the cell nucleus protecting it from pro-apoptotic signals such as TNFR1-associated death domain protein (TRADD) and tumor necrosis factor (TNF) [52]. Malignant epithelial cells deficient in K8 and K18 are approximately 100 times more sensitive to TNF–induced cell death, while K18 appears to segregate TRADD to diminish the interaction of TRADD with activated TNFR1, leading to a reduction of TNF-induced apoptosis. [52]. Despite these protective structures, the new microfluidic CTC capture devices are able to sequester cohesive cell-clusters without rupturing cell-cell adhesions.

Collective cohesive migration of epithelial cells occurs in morphogenesis, regeneration, and cancer. The cellular and molecular mechanisms underpinning cohesive migration are founded on several processes: (1) cell-cell cohesion (including the binding of α6β1 integrin to intercellular laminin), (2) collective cell polarization (into “leaders” and “followers”—Rac, β1 integrin, and PI3K are over-expressed in “leader” cells) within the clusters, and regulation of the cytoskeleton, (3) chemical and physical directional guidance, and (4) a degradation of the extracellular matrix (ECM)—partly through the action of membrane type 1 matrix metalloproteinase (MT1-MMP), which also actives MMP2—or the deposition of basement-membrane components to create a smooth scaffold and directional path between the cohesive-cluster and the ECM (reviewed in [10]).

Cells move in part as sheets, strands, and clusters resembling processes required for the development of mammary glands (reviewed in [53]). When cell-sheets or -clusters are migrating, leader cells are connected by adhesive structures, including adherens junctions, with cadherins being the primary transmembrane component of adherens junctions.

Cadherins interact with and control the actin and microtubule networks and because of their tight association with the actin cytoskeleton, adherens junctions are essential for maintaining the integrity of the migrating cell-group, therefore disrupting cadherin function seriously alters cell-cluster function [29]. Cell-cell communication enhances the capacity of cell-clusters to sense shallow gradients during morphogenesis, which is not detectable by single cells. This process is mediated by epidermal growth factor (EGF), which also plays a role in guiding the migration of mammary epithelial cells during invasive cancer growth [40]. Researchers have recently proposed that cell–cell communication via gap junctions is the mechanism of increased gradient sensitivity, which also increases the range of EGF concentrations that cell-clusters can identify within a gradient, permitting greater response to directional migratory signals [40]. Moreover, loss of E-cadherin weakens adherens junctions and allows leader cells to detach [29], possibly resulting in single-cell migration, which is where EMT is most likely to occur [10]. However, E-cadherin loss is insufficient for producing the EMT, and appears also to require upregulation of N-cadherin, vimentin, and fibronectin (reviewed in [53]). This data supports our working model that EMT in prostate cancer likely occurs in the creation of single CTCs in Gleason grade 5 tumors, rather than in the cohesive-clusters in the majority of cases.

The maintenance of the cell-clusters, including cell-cell cohesive interactions, are readily observed in the majority of invasive prostate cancers and during tumor progression, operating through a process reminiscent of embryonic tubulogenesis [11]. Interestingly, in model systems, the clusters rely on leader cells that regulate collective cell migration via Rac activation in the downstream signaling of integrin β1 and PI3K, with Rac and PI3K becoming a positive feedback loop, but β1 integrin and PI3K each regulating Rac activity independently [30]. Adhesion in gland development utilizes a novel role for E-cadherin in collective cell-cell migration, also found in epithelial dissemination [53]. For example, in mammary gland development, branching morphogenesis occurs in response to hormone stimulation and receptor tyrosine kinase signaling. Ductal elongation is accomplished by the multi-layering of a low-polarity epithelium, and polarity is reestablished as elongation ceases, dependent upon E-cadherin. While E-cadherin loss has been assigned a pro-metastatic role, recent experiments utilizing inducible knockdown of E-cadherin show cell-cell adhesion as an enabling feature for metastasis [53]. The mechanotransduction of shear stress, mediated by E-cadherin, encountered by migrating clusters is distributed over cell-cell junctions, enabling survival, and also possibly communicating the direction of movement [54].

The flexibility of the cohesive-migration response is provided by enzymes such as matrix metalloproteases (MMPs), membrane type-1 metalloproteinase (MT1-MMP), urokinase plasminogen activator (uPA, PLAU), and the urokinase plasminogen-activated receptor (uPAR, PLAUR). Many studies have implicated the serine-protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) as having special importance in cancer invasion and metastasis [55]. The increased proteolytic activity of membrane-bound and secreted proteases on the surface of cancer cells and in the transformed stroma is a common characteristic of metastatic prostate cancer. Recently, an active site-specific probe for detection of peritumoral uPAR has been created and can detect prostate cancer in bone and in soft-tissue metastases [56]. Other work has shown a “first-in-human” uPAR-PET

detection of prostate cancer for improved cancer diagnosis, staging, and individual risk stratification [57]. In addition to aggressive prostate cancer, uPAR positivity was detected in 89% (149 patients) of neoplasias at the invasive front of clusters of urothelial carcinoma of the bladder. Further, uPAR positivity was significantly associated with T-stage as well as grade and, in a univariate analysis, the uPAR group had a shorter overall survival [58]. The uPAR/uPA orchestration of pericellular proteolysis in tumor invasion is significantly increased in patients with advanced prostate cancer [59]. Pericellular proteolysis includes the production of a tumor-specific adhesion receptor that is a laminin-binding integrin variant, a novel form of α6 integrin, called α6p, created by uPA-dependent cleavage of the laminin-binding domain from the surface of tumor cells [60].

3.5. The cohesive phenotype and the laminin-binding integrins

Laminin-binding integrins (LBIs) are adhesion receptors required for the stability and structural integrity of the skin and simple glandular epithelium. There are four known laminin-binding integrins comprised of the heterodimers α3β1, α6β1, α6β4, and α7β1 [61]. The LBIs are uniquely responsible for withstanding mechanical and shear stresses, and mutations within the α3 and α6 LBI-axis result in blistering diseases of varying severity (reviewed in [62]). Recent work shows the functional role of one LBI (α6β4) as a mediator of endothelial cell protection in the setting of excessive mechanical stretch relative to lung injury [63]. LBI expression patterns have clinical significance for several epithelial-derived malignancies, for example the α6β1 integrin receptor is conserved in prostate cancer [39], is expressed on prostate tumor cells undergoing PNI [13], and acts as a marker in the aggressive phenotype of tumor cells during cancer progression [14,32,60,64]. The LBIs are especially associated with the invasion and metastasis of human prostate cancer, traversing through muscle [39,65–69].

Using TCGA data sets, copy number variations of the laminin-binding integrin axis genes are significantly increased in distinct epithelial subtypes and predict survival in bladder, cervical, and endocervical adenocarcinoma [61]. In human prostate metastatic lesions, including bone [22], these integrins are persistently and uniformly expressed in the tumor clusters (Fig. 3), independent of genetic composition. Integrin staining is found between tumor cells indicating a role in cell-cell adhesion consistent with patterns observed in early embryonic development [70]. The tumor clusters also express cytokeratin 8 and 18 (data not shown). The uniformity of integrin expression in prostate tumor cohesive-clusters (Figs. 1 and 3) is in contrast to the known molecular heterogeneity of tumor markers (e.g. Ki-67, p53, Her2, ER, or PR) in breast tumor clusters shown in Fig. 4.

Taken together, this data suggests that uniformly expressed integrins on cohesive-clusters of tumor cells may offer a uniform target, in contrast to the other molecular targets that are non-uniformly expressed. We and others have shown in pre-clinical models that blocking expression or function of α6β1 integrin curtails invasion and bone metastasis of tumor cells both *in vivo* and *in vitro* [68,71,72]. In addition, α6β4 integrin acts as a tumor-growth suppressor dependent on β4-mediated recruitment of plectin to the plasma membrane; however, in the absence of plectin, α6β4 works with Ras to stimulate tumor growth, dependent on strong activation of the Erk pathway [73], which provides a link between

overexpression of α6β4 integrin, aggressive tumor actions, and a poor prognosis [Table 2] [74]. In recent clinical studies, elevated α6 integrin expression is predictive of prostate cancer biochemical recurrence, is independently predictive of local recurrence, and is associated with bone metastasis progression, clinically detectable metastasis, and disease-specific death [81]. Increased expression of α6 integrin in bone marrow is indicative of non-aggressive prostate cancer [82], perhaps due to the growth suppressor role of the β4 integrin as seen in model systems [77].

One mechanism of LBI dynamics involves the production of a uPA/uPAR-dependent integrin variant called integrin α6p. The variant is tumor-specific and created as a post-translational modification to remove the ligand-binding region of the integrin on the cell surface (reviewed in [83]). It is currently unknown how the variant participates in cohesive tumor clusters and whether the co-localization of uPAR and α6 integrin in prostate cancer tissue would reveal aggressive subclasses of Gleason grade 6 (3+3) tumors. We have shown that preventing α6p production in prostate tumor clusters within the bone arrests bone lesion progression, resulting in curative-type lesions [64]. Considering that approximately 85% of patients with advanced disease develop bone metastases, preventing α6p production may represent a novel, non-cytotoxic treatment for prostate cancer patients with advanced disease and extensive skeletal involvement; alternatively, blocking the function of the laminin-adhesion receptors can stimulate curative-type bone metastasis lesions [64].

While our understanding of the roles adhesion molecules play in transcriptional pathways driving metastatic, epithelial cancers has increased substantially in recent years, especially with the discovery of cohesive tumor clusters, a lack of understanding persists in our ability to identify tumor specific, actionable targets for metastatic inhibition.

4. Discussion

A brief outline of the processes we have defined as the cohesive metastasis phenotype in prostate cancer would include: development of cohesive tumor-cell clusters as intraepithelial neoplasia; collective invasion into surrounding tissues; migration of cohesive-clusters through muscle to exit the gland via perineural invasion; movement of cohesive tumor-clusters (microemboli) within the circulation; and dissemination of tumor cells, as cohesive-clusters, into distant metastatic sites.

The cohesive metastatic phenotype in prostate cancer likely involves the LBI-axis and other adhesion molecules known to be active in cell-cell adhesion. For example, E-cadherin is present in prostate cancer cohesive-clusters in metastatic disease [11]. Coordination and interdependence of cadherin and integrin adhesions has been proposed to include an interdependent network crucial for cellular responses to adhesive environments (reviewed in [84]). Identification and characterization of specific biomarkers associated with transition to a collective metastatic phenotype could be a defining moment. For example, the knowledge that the cohesive metastasis phenotype is prevalent in therapeutic resistance can be used to screen for phenotypic reversal agents.

Among the LBI interactive protein partners, structural adapter proteins such as plectin are highly overexpressed in a variety of epithelial tumor types, including prostate cancer [61]. Plectin copy number amplification has significant co-occurrence with other protein members of the LBI signature. Plectin depletion in experimental systems dramatically altered adhesion structures and intermediate filament branching lengths without affecting their turnover [85]. Loss of plectin also regulates nuclear mechanotransduction in epithelial cells since nuclear deformation was increased using micro-patterned surfaces to precisely manipulate cell shape [86]. These data are consistent with the idea that increasing expression of a structural linker that is important for maintaining biomechanical strength properties and flexibility would likely be required to survive micro-environmental switches during metastasis. As stated earlier, cell migration incurs substantial physical stress on the nuclear envelope and requires efficient DNA-damage repair for cell survival [50].

Protein-protein interactions, critical for dictating cellular phenotypes, are conditional interactomes conferring flexibility of response to the changing environments during the metastatic cascade [87] and may offer tumor-specific targets. Failure to establish functional cell adhesions, and thus the assembly of associated cytoplasmic or nuclear scaffolding and signaling networks, can have severe pathological effects (reviewed in [88]). The molecular antecedents of these pathological outcomes may not be immediately predictable based on *a priori* knowledge of integrin interactome components, and thus there is a key role for discovery-based mass spectrometry-based proteomic techniques linked to highly multiplexed imaging of tumor clusters to gain new insight into integrin signaling dynamics [89,90].

Recent advances in mass spectrometry-based techniques, in particular “next-generation” Data Independent Acquisition Mass Spectrometry (DIA-MS) [91,92] and integrated bioinformatics platforms (such as The Cancer Genome Atlas, or TCGA) [61], now enable sensitive discovery and quantitative monitoring of binding partners across a large number of experimental conditions and replicates. The ability to recover protein complexes downstream of the laminin-binding integrins (LBI) in cohesive-clusters of prostate tumors rapidly, under native conditions, and with an increased sensitivity [92], will lead to robust analysis of LBI proteome dynamics in prostate cancer metastases. Current proximity ligation assays are capable of detecting the biomolecular protein-protein interactions utilizing lysine-linked fluorophores in tissue [93]. This technological advance, coupled with the new knowledge that cohesive tumor clusters utilize adhesion molecules for aggressive dissemination, will likely be translated into predictions of drug efficacy and sensitivity. The potential for combinatorial treatments to improve upon patient outcome may also be a viable option with discovery of such targets. Although research in proteomics has led to multiple discoveries of potential protein biomarkers, there are only nine cancer protein biomarkers that are currently approved by the FDA—others have not had specific follow-up validation studies for clinical use [94].

There is a compelling clinical need to include the testing of anti-metastatic therapies, along with systemic chemotherapy approaches, for epithelial cancer treatment [21]. An alternative strategy also is required to tailor therapies to prostate cancer that requires aggressive treatment—avoiding both expensive and high-morbidity approaches when possible [20]. For

example, as secondary prevention steps, early blockage of skeletal-related events (SREs) and their complications includes preventing early invasion and successful colonization of bone [60,95], stimulating the host response to bone lesions [64], preventing dormant tumors from escaping the bone [21], and identifying bone health as a preemptive determinant of secondary prevention [96]. Recent results indicate that while early initiation of zoledronic acid (ZA) therapy for patients with castration-resistant prostate cancer and bone metastasis significantly reduced skeletal complications [97] and pain [98,99], it was ineffective for the prevention of bone metastases in high-risk localized prostate cancer patients [100]. Considering current research indicates that prostate cancer can disseminate early to bone metastatic sites [21,23], and recurrent CRPC or NEPC will aggressively disseminate, more work needs to be done to detect or prevent transition to the metastasis phenotype in prostate cancer.

5. Conclusions

Our understanding of the biophysical and tissue-based physiology parameters of prostate cancer metastasis is advancing. The use of new technology and integrative bioinformatics (e.g. TCGA data and NCI genomic data commons) can be employed to define essential protein components contributing to the cohesive metastatic phenotype and clinical outcome. The greatest challenge in targeting cohesive clusters to prevent or treat metastasis is to utilize current 3D screening methods combined with metastasis end-point analysis to generate candidate molecules. Changing the current high throughput screening endpoints from cell killing to altering the cohesive cluster metastasis phenotype would be a major advance. In addition, there is also a need to develop FDA-approved CTC-cluster isolation tools in order to assist precision medicine type discovery of clinically relevant molecular features of unique CTC-clusters within a given patient. New strategies are likely to emerge with pathways that discriminate aggressive versus indolent disease with an aim toward providing information useful for choosing treatment options, including active surveillance. Candidate pathways for adjuvant treatments also may be discovered to overcome CAM-DR or CAM-RR, well-known impediments to therapeutic responses.

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Abbreviations

ADT	androgen-depletion therapy
AR	androgen receptor

BPH	benign prostatic hypertrophy
CAM-DR	cell adhesion-mediated drug resistance
CAM-RR	cell adhesion-mediated radiation resistance
CD49f	alpha-6 integrin sub-unit
CK	cytokeratin
CTC	circulating tumor cell
DTC	disseminated tumor cell
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ER	estrogen receptor
FGFR	fibroblast growth factor receptors
Her1	human epidermal growth factor receptor 1
Her2	human epidermal growth factor receptor 2
LBI	laminin-binding integrin
mCRPC	metastatic castration-resistant prostate cancer
MET	mesenchymal-to-epithelial transition
METS	metastasis
MMP2	matrix metalloproteinase-2
MT1-MMP	membrane type-1 matrix metalloproteinase
NE	nuclear envelope
NEPC	neuroendocrine prostatic cancer
PCa	prostate cancer
PI3K	phosphoinositide 3-kinase
PNI	perineural invasion
PNS	peripheral nervous system
PR	progesterone receptor

PSA	prostate-specific antigen
SKE	skeletal-related event
TAM	tumor associated macrophages
TCGA	The Cancer Genome Atlas
TNF	tumor necrosis factor
TRADD	tumor necrosis factor receptor 1-associated death domain protein
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen-activated receptor.

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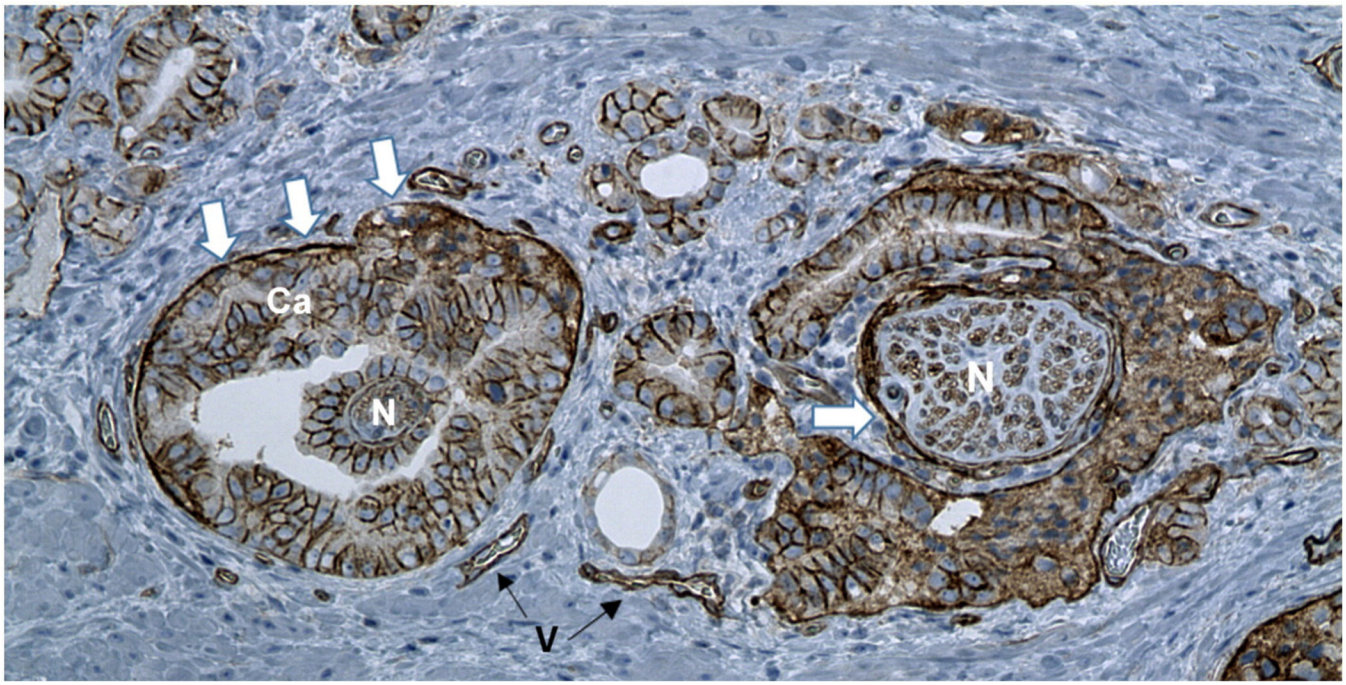

Fig. 1. Integrin α6 Expression in Endoneural and Perineural Invasion in Human Prostate Cancer

During tumor invasion on prostatic nerves, human prostate tumor cells express the α6 integrin stained with the AA6NT polyclonal antibody specific for the α6 integrin (brown). The nerves (**N**) are surrounded by a perineural sheath (**white arrows**). Left nerve contains endoneural invasion by cancer and right nerve contains perineural cancer distribution. Note the clusters of tumor cells and the absence of cancer cell (**Ca**) invasion along vessels (**V**) when compared to significant invasion of the nerve. α6 integrin is expressed in endothelial cells within vessels as expected.

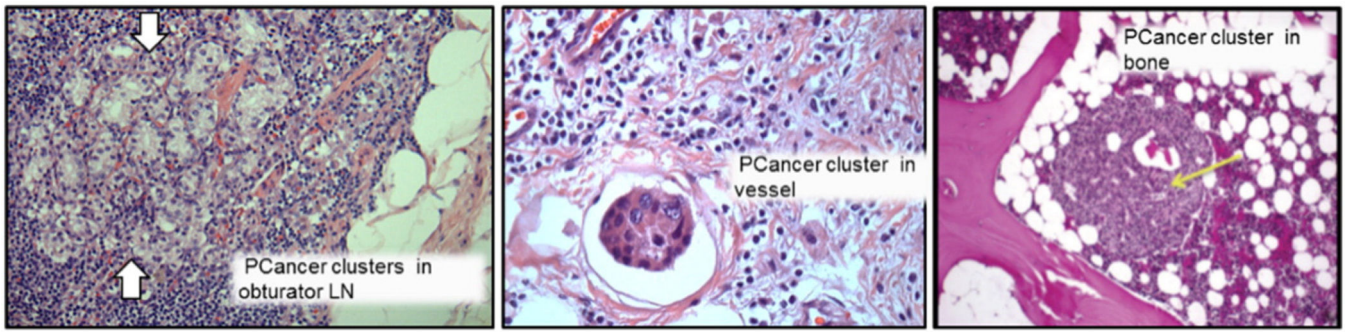

Fig. 2. Human prostate cancer clusters in human tissue

De-identified samples from prostate cancer patients from a pelvic lymph node (**left panel**), prostate tumor tissue (**middle panel**), and in bone (**right panel**) were fixed and stained with Hematoxylin and eosin. The presence of prostate cancer clusters are observed in the obturator lymph nodes (**left panel**, between white arrows), within vessels (**middle panel**) and within the bone marrow (**right panel**, yellow arrow).

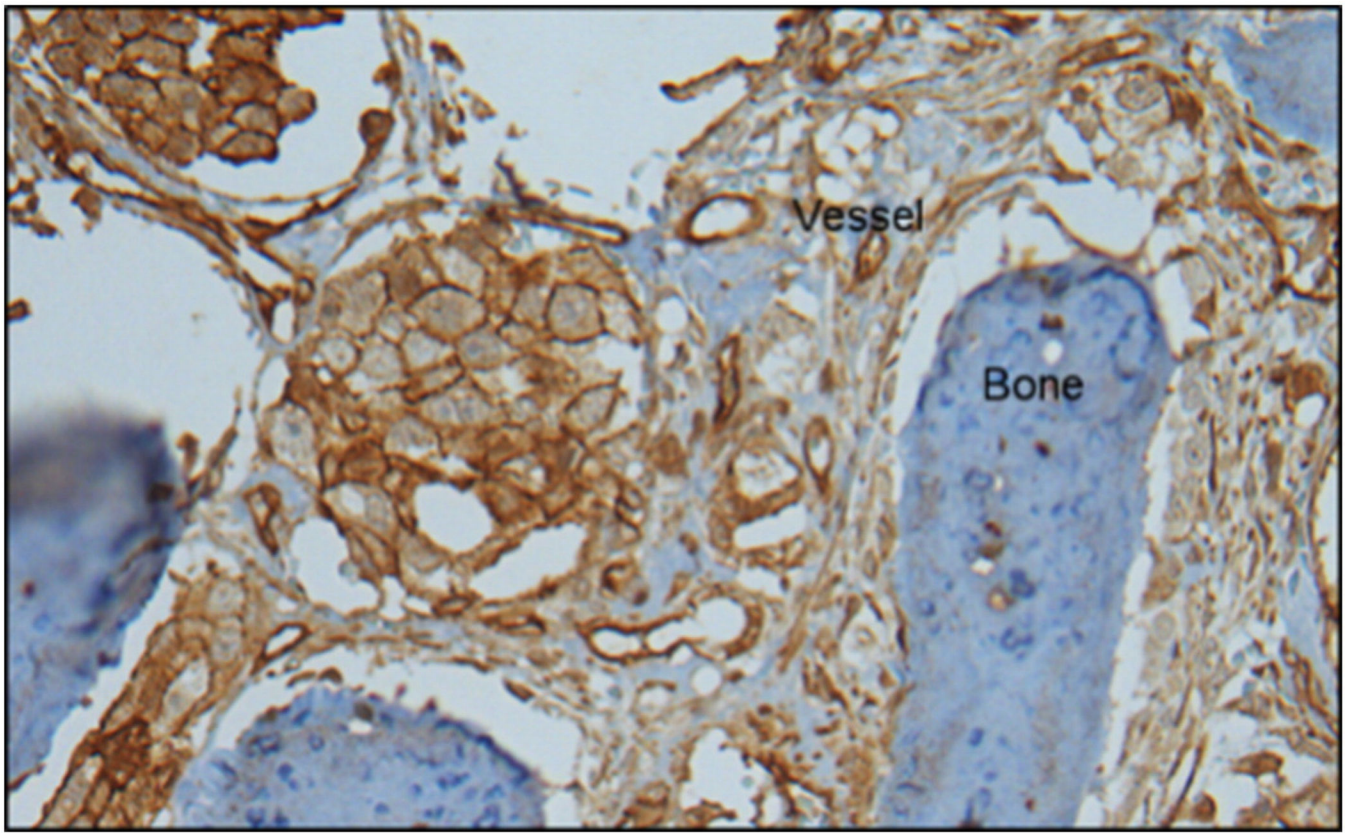

Fig. 3. Interosseous metastasis showing cell-cell adhesion as detected by α6 integrin (CD49f) specific staining

Histological section containing bone (**Bone**), α6 positive vessels (**Vessel**) and a cohesive cluster of human prostate cancer, with a cell-cell adhesion distribution of the α6 integrin.

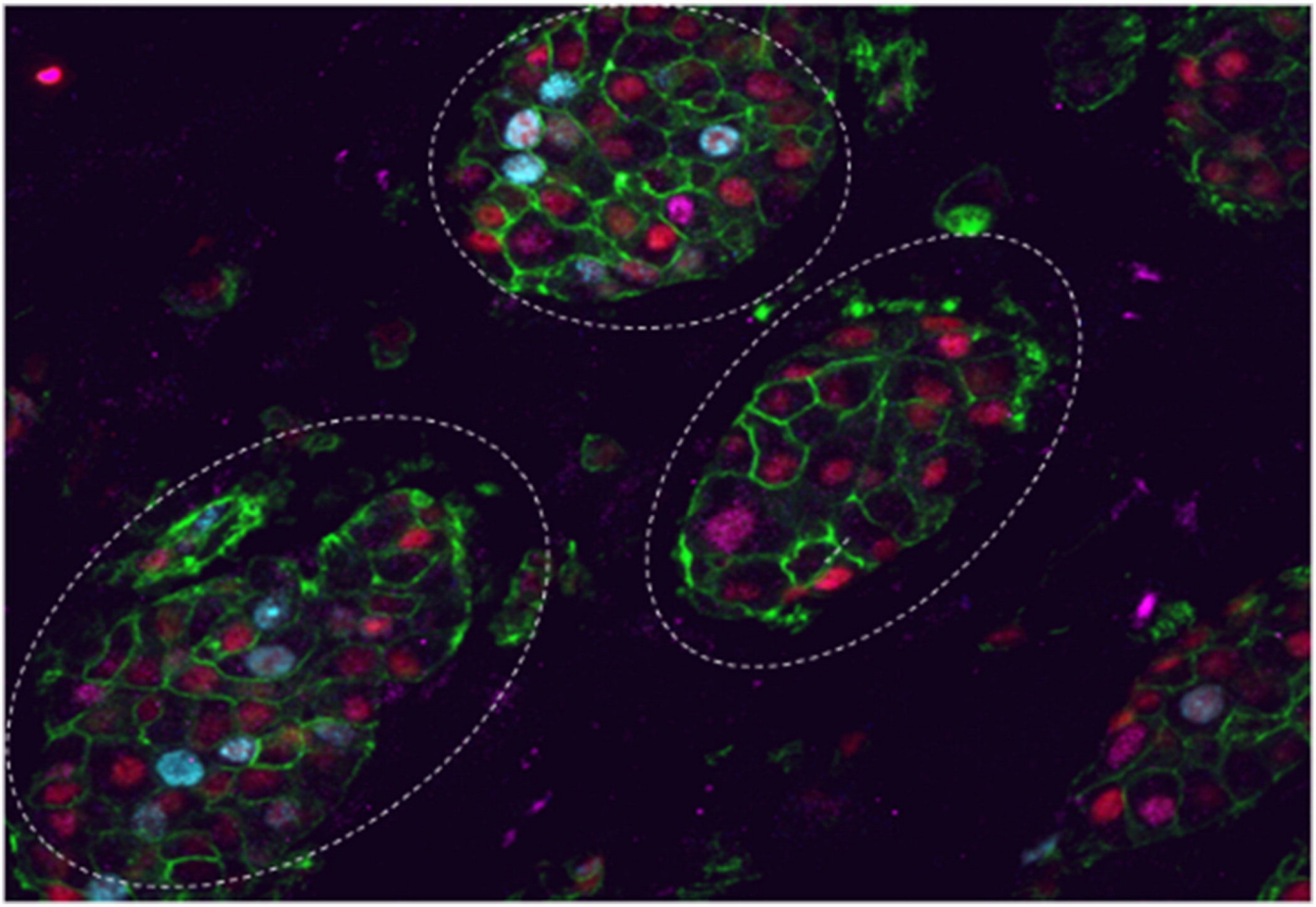

Fig. 4. Clusters of human breast carcinoma demonstrating molecular heterogeneity using immuno-histochemistry

Five distinct haptens were detected including ER (QD 585, red), Ki-67 (QD 605, light blue), PR (QD 625, blue), p53 (QD 655, yellow) and Her2 (QD 705, green).

Table 1

Cell-cell adhesion clusters in human prostate cancer tissue and metastases.

Tumor Location/ Detection	Major findings	Ref.
lymph nodes, bone, adrenals, liver, bladder, sacrum, blood whole genome sequencing	In 5/10 cases clusters of mutations presented subclonally across multiple metastases, suggesting polyclonal seeding between organ site metastasis; often occurs as a spread between distant sites, not as separate invasions from the primary tumor	[4]
bone, lymph node, biopsy and tissue microarrays Immunohistochemistry	Clusters >30 = EpCAM-high METS, <30 = EpCAM-low METS; ↑ EpCAM expression occurs early in PCa (GSC ≤ 7 including 3 + 4), in GSC ≥ 7 (including 4 + 3), and in METS; mesenchymal PCa cells express no/low levels of EpCAM vs. epithelial PCa cells; no observed effects on PCa cell proliferation w/ EpCAM downregulation (long term or short term)	[41]
Needle Biopsy samples Immunohistochemistry	CD49f + (α6 integrin), Trop-2+, CD24- subset: CK5 (basal cell marker) is ↑ and p63, CK8/18, AR are ↓. CD49f–Hi cells overexpressed genes from the NOTCH, FGFR, and WNT development pathways. Paraclones = irregular, loose colonies, 32–100 cells; Mesoclones = larger, irregular, and loose, scattered cells w/ different morphology, 100 to 500 cells; Holoclones = round-shape, dense, cells with different morphology and with good mutual connections, >500 cells	[9]
bone, lymph node, lung, liver CTCs in blood	Mean survival in patients ≥4 CTCs / 7.5 cm^3 blood was 8.4 months vs. 15.1 months for all 100 patients; patients <4 CTCs / 7.5 cm^3 blood had better survival, but median was unavailable (due to high censoring); mean CTC count in patients alive vs. deceased after 20 months = 12 vs. 294 [median (range), respectively: 1 (0–117), 29 (0–2572)]	[34]
Needle Biopsy and Radical Prostatectomy samples Immunohistochemistry	Identified 3 phenotypes - expressing α6 and α3 integrins, but not co-localized (type I), α6 integrin only (type II), or α3 integrin only (type III). *In situ* hybridization and DNA analysis showed genetic differences in multiple tumors from same prostate	[39]

Table 2

Cell-cell adhesion is important in malignant progression, using model systems.

Model/Cancer	Molecules	Mechanism	Tumor Progression	Ref.
Mouse/PCa	β4 integrin, ErbB2, c-Met	β4 promotes prostate tumorigenesis by amplifying ErbB2 and c-Met signaling in tumor progenitor cells	β4 signals promote tumor progenitor cell self-renewal, growth of transit-amplifying tumor cells	[75]
Mouse/Skin	α6β4 integrin, TGFβ, α6 and β4 subcloned w/ involucrin promoter (Invα6β4)	α6β4 promotes carcinoma invasion by activation of PI3-K; α6β4 signals to the Ras-MAPK (mitogen-activated protein kinase) pathway; α6β4 enhances basal cell growth *in vivo* and in culture, and overcomes TGFβ-mediated growth inhibition in culture	Invα6β4 mice developed 3–4× more SCCs than wt, while 100% of Invα6β4 mice developed SCCs vs. only 40% of wt	[76]
Mouse/Skin	α6β4 integrin, plectin	α6β4 mediates tumor growth suppression dependent on β4-mediated recruitment of plectin to the plasma membrane	with Ras expression in mTICs, α6β4 works with Ras to stimulate tumor growth, and needs strong activation of the Erk pathway	[73]
Mouse/Skin	α6β4 integrin	α6β4-positive cells correlate to a larger supra-basal proliferative layer; K8 is found in α6β4-positive cells in the proliferative compartment of high-risk tumors	distribution of α6β4 integrin complex indicates risk of malignant progression in experimental skin carcinogenesis	[77]
Human/PCa	(CD49f) α6 integrin, basal stem cells	CD49f–hi (α6) cells overexpress many genes found in the NOTCH, FGFR, and WNT development pathways; CD49f–lo cells overexpress genes associated with prostate luminal cells or prostate cancer, including AR, KRT8, KLK3, NKX3–1, TMPRSS2, and AMACR	hormone-sensitive metastatic samples showed higher enrichment for CD49f–hi gene signature; SCNC had higher CD49f–hi signature scores than other phenotypes	[78]
Mouse/PCa	CD26, basal cells	combination of c-Myc overexpression and activation of PI3K/AKT pathway drives high-grade PCa derived from basal cells; the same oncogenic stress drives low-grade PCa derived from luminal cells	distinct PCa subtypes may arise from luminal and basal epithelial cells experiencing similar oncogenic insults	[79]
PCa cell lines	cell lines: bone (PC3), brain (DU145), lymph node (LNCaP)	when embedded in a BME gel basement membrane, cancer cells can grow as spheroids and aggregate forming larger and larger structures	only the PC3 cells form aggregates of clusters, confirming their aggressive potential	[80]