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How to be good at being bad: centrosome amplification and mitotic propensity drive intratumoral heterogeneity

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Abstract

Cancer is truly an iconic disease—a tour de force whose multiple formidable strengths can be attributed to the bewildering heterogeneity that a tumor can manifest both spatially and temporally. A Darwinian evolutionary process is believed to undergird, at least in part, the generation of this heterogeneity that contributes to poor clinical outcomes. Risk assessment in clinical oncology is currently based on a small number of clinicopathologic factors (like stage, histological grade, receptor status, and serum tumor markers) and offers limited accuracy in predicting disease course as evidenced by the prognostic heterogeneity that persists in risk segments produced by present-day models. We posit that this insufficiency stems from the exclusion of key risk contributors from such models, especially the omission of certain factors implicated in generating intratumoral heterogeneity. The extent of centrosome amplification and the mitotic propensity inherent in a tumor are two such vital factors whose contributions to poor prognosis are presently overlooked in risk prognostication. Supernumerary centrosomes occur widely in tumors and are potent drivers of chromosomal instability that fosters intratumoral heterogeneity. The mitotic propensity of a proliferating population of tumor cells reflects the cell cycling kinetics of that population. Since frequent passage through improperly regulated mitotic divisions accelerates production of diverse genotypes, the mitotic propensity inherent in a tumor serves as a powerful beacon of risk. In this review, we highlight how centrosome amplification and error-prone mitoses contribute to poor clinical outcomes and urge the need to develop these cancer-specific traits as much-needed clinically-facile prognostic biomarkers with immense potential value for individualized cancer treatment in the clinic.

Keywords

Mitotic propensity; Centrosome amplification; Intratumoral heterogeneity; Cancer; Biomarker; Prognostic

1 Prologue

“The Dark Arts are many, varied, ever-changing and eternal. Fighting them is like fighting a many-headed monster, which, each time a neck is severed, sprouts a head even fiercer and cleverer than before. You are fighting that which is unfixed, mutating, indestructible.”

—Severus Snape, Harry Potter and The Half Blood Prince.

For cancer patients and their families, Professor Snape's hopeless depiction of the struggle of Good against the Dark Arts seems unnervingly familiar. Though treatments are much improved, cancer—the malady affecting one in three of us—is rarely cured and seems to use its own increasingly “fiercer and cleverer” dark forces against us. Although “cancer” was previously perceived as a single disease, some kind of aberrant lump or rogue collection of circulating cells, cancer biologists are now beginning to recognize the reality of this “many-headed monster” and the shortfall in our understanding of the multiple, shape-shifting personalities of this “evil” force.

It has been long appreciated that cancer's malignant traits arise by way of stochastic mutations in the genomes of normal cells. These mutations, ranging from single point mutations to whole chromosome aberrations, collectively bring about gains of function in oncogenes to further a tumor's malevolence, as well as losses of function through mutations in tumor suppressor genes to downregulate or silence processes that are essential to keep the cell from becoming cancerous [1]. In their compilations of the “hallmarks” of cancer, Hanahan and Weinberg [2] identify eight traits (reimagined herein as personality traits) possessed by all cancers that help the tumor to “be good at being bad”:

1. *Cancer is self-centered.* To advance its destructive mission, it has the ability to remain constitutively “switched on” in response to growth signals.
2. *Cancer is disobedient.* It has the ability to resist being “switched off” by inhibitory growth signals.
3. *Cancer is persistent.* It circumvents usual checkpoints to control cellular growth and acquires limitless potential to reproduce.
4. *Cancer defies death.* Whereas normal cells with faulty genetics are rapidly consigned to programmed cell death, cancer cells can avoid apoptosis.
5. *Cancer has bad blood.* It can “sprout” a blood supply around and within the tumor to “feed” on the body like a parasite.
6. *Cancer is destructive.* It can disrupt, invade, and overcome normal tissues, as well as form metastases to spread its evil to multiple locations in the body.

7. *Cancer is deceptive.* It can evade immune surveillance and subsequent destruction by the immune system through complex cloaking devices.
8. *Cancer is a hacker.* It can change the code and reprogram energy metabolism to harness a force for evil.

Clearly, cancer is not a readily predictable character: the more we explore his complex traits, the more we see his propensity to acquire new powers, to hone his talents, and to develop new methods of attack. For instance, we have only recently become familiar with how cancer's cells amass extra centrosomes and via improper mitotic divisions, produce a cascade of errors that allows him to expand his forces, diversify his armamentarium, and make him invincible. The deeper we probe, the more we understand that there is much left to understand.

It is convenient to think of cancer's development as a stepwise progression through a series of linear stages: birth, through character building life events, to the eventual death of the tumor - frequently taking the patient with him. However, there is increasing evidence to suggest that his development is an ebb and flow; swinging between flurries of intense activity and fluctuation of cell phenotype, followed by periods of relative quiescence and even regression whereby mutant cells may cluster with regular cells and function ostensibly "normally" [3]. Though cancer's victims tend to simplistically believe him to be a singular villain against which we simply have not yet found the right kryptonite, it has long been suggested [4, 5] that cancer evolves via a Darwinian evolutionlike process that is almost a throwback to the dawn of multicellular life—into a population of deviants. These deviants all possess certain traits in common and are all defective with respect to regulation of cell proliferation and homeostasis; however, there is wide and unpredictable spatial and temporal variation among them [6]. Thus, cancer is legion, for he is many - and these malignant clades of cells, each inhabiting a distinct microenvironment, are often in competition and cooperation with each other [7].

Recent research advances have provided strong evidence supporting the existence of dynamic intratumoral heterogeneity (ITH); for example, next generation sequencing (NGS) [1] and genomic studies of biopsies taken at different time-points, and in different tumor regions [8]. ITH appears to be a requisite for cancer's survival; this idea stems from the concept that one area of tumor cells, being spatially and genetically distinct from other areas, may have a competitive advantage when placed against the dynamic background of the patient's homeostatic mechanisms, their responsive immune system, or therapeutic interventions.

Nurturing to life the idea of Darwinian tumor evolution, Yap et al [9] propose the analogy of a "Darwinian tree model" of tumor growth, suggesting that this way of thinking may aid researchers in their quest to not only identify but also validate predictive cancer biomarkers. In this model, the trunk of the tree represents the collection of ubiquitous mutations driving changes common to all cells within cancer, while branches represent spatially separated tumoral regions in which heterogeneous mutations exist that are not present in the "trunk" cells. Secondary, tertiary and increasingly divergent "branches" of the tree provide a useful metaphoric representation of the potential for different cell subgroups to exist within a single

population of tumor cells; the more “branches” and “leaves” relative to the core population of trunk cells, or the most common recent ancestor, the more cancer is karyotypically diverse [9]. Regions of localized hypermutation—termed “kataegis” after the suitably dark and brooding Greek word for “thunderstorm” [10]—are typically found in breast cancer susceptibility genes. This wide variation in genotype and phenotype (1) makes cancer harder to defeat as traditional single-tumor biopsies used to assess the genomics of a given lesion, and on which intervention decisions and treatment plans are made, may not representatively reflect cancer's true personality or personalities, and (2) helps the many-headed beast be better at being bad, further accounting for high treatment failure rates and poor patient outcomes.

Researchers are only now beginning to comprehend (1) how cancer acquires the ITH that allows a tumor to grow, invade, and spread, (2) how this genetic diversity is generated, (3) the full biological relevance of such variation, and (4) the relationship between mutational processes and clonal expansion [8]. To add further complexity to this problem, other factors contributing to the tumor's landscape must also be considered in this struggle of good versus evil. For example, epigenetic controls are likely to be important, as is dissection of the tumor-associated stroma in which apparently normal, healthy cells are press-ganged into the tumor microenvironment, unwittingly playing a part in furthering cancer's intent and culminating in their own demise.

In this article, we discuss the idea that genetic mutations and other cellular processes leading to amplification of centrosomes underpin chromosomal instability, with a high propensity to undergo error-prone mitoses as the vehicle driving genetic diversity within cancer's legion of dividing cells. These disregarded drivers of ITH may be promising biomarkers for earlier or more precise diagnoses, more accurate prediction of disease progression, patient response to specific therapies and patient outcomes, and may perhaps yield new ways to overcome the villain, or villains, that are cancer.

To flesh out cancer's nefarious character and bring him to life for the reader, we present his persona, misdeeds, and a game-plan to combat him more effectively in two acts: in act one, we discuss the process surrounding the birth of this rogue who rapidly evolves multiplexed wickedness through the agency of the “evil twin” traits of centrosome amplification and mitotic propensity. In act two, we ponder how the cunning devices used by cancer to establish his reign can perhaps be exploited as biomarkers predicting his destructive behavior.

2 Act one: cancer's cache of arms

2.1 Scene one

2.1.1 The birth of Machiavelli: origin of intratumoral heterogeneity—Folklore, stories, and even real life attest that villains are rarely “born evil”; they most often become that way, for example, through neglect, abuse, brainwashing, or dark magic. Although we recognize that some patients have a hereditary, genetic predisposition to certain cancers, there is still a point in time at which the Machiavellian switch from normal to malignant cell occurs. The point at which this transformation is initiated varies greatly between different

types of cancer and in different patients [7]. Understanding the mechanisms behind this switch is a key to our understanding of disease progression and outcome.

Once upon a time, more than 100 years ago, arose the concept that our maleficent protagonist, cancer, might be able to originate from a stem cell. Increased interest in this idea was generated in the 1950s and 1960s when some scientists suggested that a normal adult stem cell might be “turned to the dark side” because of a chance mutation acting as a molecular switch. This random change could render the stem cell tumorigenic—capable by itself of forming a new lesion through linear clonal expansion. When faced with a challenge to tumor progression, such as chemotherapy, the stem cell model hypothesizes that the malignant stem cell is able to autonomously regenerate, taking a different route towards rapid expansion of mutated cells. It was thought these malevolent, “Time Lord”-like cells might be at the root of all tumors and may cause cancer's recurrence after remission. Indeed, high expression of stem cell markers correlates with worse clinical outcome for patients, suggesting the importance of this mechanism in tumor progression [6]. Molecular studies, however, have shown this view to be somewhat simplistic. The clonal evolution model explains that genomic instability is at the heart of ITH and increasing tumor aggressiveness. In other words, multiclonal progression through the continuous saltatory acquisition of new somatic changes that, for cancer, are of ruthless, self-serving benefit [7] at a given point in time or space, can lead to his increased genetic diversity and rapid evolution of phenotypes superiorly adapted to specific microenvironments.

The stem cell model and that of clonal tumor evolution are not mutually exclusive. It is commonly accepted that tumors arising from cancer stem cells may have increased treatment resistance and a higher capacity for metastasis [6]. For our villain to successfully evade and even acquire resistance to the human immune system and therapeutic interventions, he must stay one step ahead of the game; to revert to a stem cell and effectively start again when an insurmountable challenge is presented seems counterintuitive, but may be just one weapon in his arsenal that allows him to rear another, stronger, uglier head. For a “bad guy” to be the best at being bad, he needs diverse superpowers and may utilize both models of progression, at once, in tandem, or separately, to advance his villainous plan.

3 Scene two

3.1 Engendering Jekyll, Hyde, and everyone in between: centrosome amplification, clustering and chromothripsis

At some point in the lifetime of solid tumors, tumor cells undergo a reprogramming of cell shape, state and behavior that paves the way for an increase in invasiveness and aggressiveness [11]. Cancer suddenly cold-shoulders his old companions and friends, strategically forging new partnerships to enable his expansionist agenda; in the case of epithelial cells, this is usually manifest as (1) loss of intercellular junctions (tight junctions, adherens junctions, desmosomes and gap junctions) between tumor cells, (2) loss of apico-basolateral polarity of tumor cells, (3) establishment of heterocellular gap junctional communication between tumoral and stromal cells leading to “activation” of the tumor micro-environment, (4) secretion and activation of stromal proteases to facilitate motility of

invasive cells through the extracellular matrix, and (5) destruction of the basement membrane [12]. The extent to which cancer is able to invade healthy tissues in this manner, and resist therapies, correlates with the extent of clonal diversity [3]. This character change in Cancer occurs stochastically, driven by genome alterations, epigenetic changes and karyotype changes that change the transcriptome of his cells [13].

At the heart of this punctuated personality, makeover lies the phenomenon of chromosomal instability (CIN) which is essentially the rate of change of karyotypes within a given cell population. CIN can be either numerical CIN (gain or loss of whole chromosomes or fractions of chromosomes resulting in aneuploidy) or structural CIN (determined by structural non-clonal chromosomal aberrations). There is a mounting body of evidence suggesting that cancer's "CINful" character may be one of the earliest drivers of ITH [8, 10]. Faults in mechanisms that normally preserve genome integrity and ensure the equal partitioning of genetic material between progeny cells, for example, a loss of mitotic checkpoint function or defective kinetochore attachments, can cause karyotypic rearrangements and generate a pool of progeny cells that differ genetically from their parents.

An often-overlooked and potent driver of CIN is centrosome amplification (CA). Extra centrosomes are highly prevalent in both solid and hematologic cancers—yet they occur so rarely in normal human tissue [14]. Furthermore, amplified centrosomes are often aberrant or misshapen, observed in unusual positions within the cell, nucleate excess microtubules, and have been postulated to promote cancer cell polarization and directed migration [15].

But how does cancer acquire this illegitimate wealth? Normally, newly formed daughter cells contain only one centrosome that must be semi-conservatively replicated before the next mitotic division. But in its early development, cancer has other schemes. There are four ways that amplified centrosomes are thought to occur: (1) via failure of the cell to undergo cytokinesis, resulting in a doubling of the genome as well as the centrosomes; (2) the inappropriate replication of centrosomes, either too many replications, resulting in more than the normal complement, or replication at an inappropriate time resulting in excess centrosomes in the daughter cells. There is also some evidence to suggest that (3) the inappropriate splitting of centrioles has a minor role in generating supernumerous centrosomes, and (4) the possibility that centrosomes can arise *de novo* [14].

But how does cancer circumvent the seemingly inevitable consequence of centrosomal excess, namely, spindle multipolarity? In cancer cells, excess centrosomes do produce a transient multipolar spindle state; however, this state is quickly resolved and the centrosomes are cleverly clustered into two polar groups, giving rise to pseudo-bipolar spindles. Some slippage in the efficacy of centrosome clustering occasionally yields low-grade spindle multipolarity that might even be tolerable. In either case, merotelic attachment of individual kinetochores to more than one spindle pole is a frequent occurrence. Such inappropriate attachments can cause CIN in the resulting two, three, or more daughter cells via missegregation of whole chromosomes and/or the separation of parts of chromosomes via the stress placed on chromosomes by microtubule attachments and their misguided

forces rendering the chromosomes unstable and liable to break [14]. Cancer thus uses CA and clustering to maintain optimal rates of CIN and to spur his steady evolution [15].

Further genomic instability that cancer may use to his advantage may arise through the process of chromothripsis. In a cell with CA, where chromosomes are mis-segregated via faulty merotelic attachments, lagging chromosomes or pieces thereof can become trapped within the cleavage furrow, forming micronuclei. Newly generated micronuclei may undergo defective and asynchronous DNA replication, resulting in DNA damage and often extensive pulverization of the DNA in the micronucleus. These fragmented chromosomal segments can then rejoin to give rise to chromosomes with thousands of rearrangements arising from a single catastrophic event and can also subsequently be reincorporated into the main nucleus of a daughter cell. These rearranged chromosomes can persist for several cell cycles, often overhauling cancer's personality and promoting his evolution in dramatic and unpredictable ways [16].

CIN generated via CA has been shown to propagate existing malignancies and initiate new ones [15]. CA increases centrosomal microtubule nucleation [17], which in turn increases Rac1 activity, disrupts normal cell–cell adhesion, and promotes the invasion of surrounding tissues by tumor cells [18]. Studies in *Drosophila* have also shown that stem cell proliferation disrupted by CA can produce abnormally symmetrical daughter cells in mitosis. Furthermore, when transplanted to healthy, wild type *Drosophila* abdomens, the CA-disrupted stem cells were observed to form tumors *de novo* and even metastases [15].

The extent of CA has long been correlated with cancer aggressiveness, but these studies have been qualitative or at best semi-quantitative [19]. We believe that, in order to kick-start his imperial agenda and diversify his arsenal, our archenemy cancer must generate CIN through CA as early as possible: right at the beginning of his transfiguration from normal to abnormal cell. This is not a new proposal; Boveri surmised that CA was responsible for the malignancy of cancer cells as far back as the turn of the century [20], but his hypothesis has been gravely overshadowed by the discovery and increasingly complex analysis of chromosomes, DNA, genes, transcriptomes, proteomes, regulatory networks, and so on. Given that CA has been identified in premalignant lesions and pre-dates what is classically referred to as a grade 1 lesion [21, 22], CA can be used not just as a marker of progression once disease is already established, but can perhaps be quantitatively used in premalignancy to predict outcomes. We propose that the earlier this rapid burst of karyotypic shuffling occurs, the greater the chance that individual or small groups of subclonal cells can evolve to achieve malignant fitness. Furthermore, the fact that supernumerous centrosomes become clustered means they are inherited by progeny cells. This confers increased CIN to progeny and enables them to be even better at being bad than the parent cells.

4 Scene three

4.1 Cancer's “evil twin” traits: mitotic propensity and centrosome amplification

It is noteworthy though that while CA may be necessary, it may not be sufficient—although CA underpins the dark knight's CIN and allows him to diversify his phenotypic tools, the batmobile driving this forward is the frequency of passage through error-prone mitoses:

without mitosis, diversity cannot be achieved. Simply put, increasing the number of division cycles increases the probability of producing clones that are “more malevolent”, as cancer is a genomic disease of probability [23]. The propensity to undergo frequent mitoses can serve as a critical morphologic surrogate for the kinetics of cell cycling in the proliferative population; mitotic propensity thus illuminates a fundamental, inherent and highly risk-predictive aspect of the tumor's biology.

Increased frequency of erroneous mitoses among proliferating cells not only spreads mutations throughout the population through clonal expansion, but cancer cells accumulate new genetic changes through each round of imperfect cell division. This greatly increases diversity [7] and leads to a vicious spiral [6]. More mitoses may also result in increased shortening of the telomeres, further contributing to CIN by eroding protective DNA caps [24]; unprotected chromosome ends can engage in illegitimate recombination, resulting in end-to-end fusion cycles and unstable dicentric chromosomes culminating in more karyotypic shuffling. Cancer treads a tightrope here though because he needs to attain just the right level of aneuploidy required to be good at being bad; high-grade aneuploidy could render the cell inviable and thwart the tumor's growth and dominance agenda.

Together, CA and rapid mitotic turnover early in his life allow Cancer to rapidly generate a population of genetically diverse cells to go forth as subclones and achieve the overarching aim of the disease: to become a supreme power—an invasive, evasive, and aggressive tumor. We believe it is advantageous for cancer to possess as varied a genetic reservoir as possible and, through error-prone mitoses, CIN must persist in continuing to expand the phenotypic pool. Although the specific mutations that lead to CA are not well defined, it seems clear that CA, coupled with mutations leading to the deregulation of proliferation, are relatively early events in the tumor's life history. Evidence from the Breast Cancer Working Group of the International Cancer Genome Consortium consistently finds that the most common recent ancestral cell, to which other tumor cells can be traced back in lineage, appears surprisingly early in molecular time [8]. This suggests that nascent cancer cells invest a lot of resources in generating subclonal diversity. Furthermore, several known driver mutations associated with CIN appear in many breast tumors at an early stage of development [8].

5 Scene four

5.1 Dodging the defense: cancer deceives and evades the host immune system

By evolving into a brew of diverse entities very early in his life, cancer has just the right weapons to be able to respond and adapt to new and continuing nemeses from the dynamic environment of the human body; for example, interactions with differing, normal cell types, the tumor's inefficient vasculature leading to varying oxygenation and acidosis levels throughout the lesion [6, 7], and later, to challenges stemming from therapeutic interventions. Most malignant tumors are antigenic to some extent in the host, and in most cases, the host immune system does mount some responses; which, depending on the patient's immune status and clonal composition of the individual cancer, can have different outcomes: elimination (where tumor cells are recognized based on specific surface antigens and gotten rid of), equilibrium (where the cancer is not completely eliminated but does not

progress or metastasize), or escape (where the cancer cells that can resist antitumor immune responses are selected for, leading to disease progression) [25]. In order to tip the scales in his favor and ensure his own survival, cancer uses sophisticated means to cripple the host's immune system and defy detection and rejection. Some of his means include (1) dodging surveillance mechanisms by shutting down tell-tale molecules on the cell surface that normally alert the immune system to attack them, (2) aggressively suppressing killer T-cells and making them too sluggish to recognize renegade tumor cells and touch off a full-scale immune response, (3) producing proteins that attract immature immune cells and tricking these cells into becoming immunosuppressive, (4) producing proinflammatory cytokines that result in a state of chronic inflammation and driving inflammatory cells to cloak and protect tumor cells from immune attack, and (5) becoming resistant to complement system and to lysis by T-cells or natural killer cells.

Through the counter-attack of CIN-driven ITH, cancer thus craftily ensures self-preservation in the face of seemingly insurmountable challenges and even takes the bite out of therapy.

6 Scene five

6.1 Breaking bad: the switch from mitosis to metastasis

Thus, far we have described how our villain may commence his evil agenda: to rapidly acquire enough genetic diversity so as to have a wide variety of malicious traits to choose from when selecting those that will help him to overcome his nemeses. This not only allows the tumor to evade the immune system and grow larger in size, but through the proliferation of cells with high CIN, driven by CA and high mitotic propensity, allows continued adaptation so that cancer may thrive.

At some point, however, our villain shifts attention to a completely different agenda. Having successfully inhabited a physiological niche, and perhaps even co-opted normal cells into the tumor microenvironment to help evade the immune system, the tumor becomes set not just on gaining mass, but on achieving “world domination”. No longer content—or able—to remain hidden from the body, acquiring evil traits and sapping strength from the host, the tumor must switch from a growth and diversification agenda, to a migratory one. What causes this switch from a division-focused agenda to one centered on dissemination, and when in the tumor's life this inflexion point occurs, are still relatively uncharted territory, but the literature provides some useful ideas.

Laws of ecological equilibrium dictate that beyond a certain point, and assuming no major shifts in the tumor's environment, frequent error-prone mitoses may in fact compromise the fitness of the clones being generated. This may be due to progressively decreased fidelity in the transmission of a functional genome. Indeed studies suggest the intolerance of excessive CIN in carcinomas [26]. It has also been proposed that there may be an optimal level of CIN for tumor progression [27] beyond which further instability confers no growth advantage and may even be deleterious for cancer cell survival [27-30]. This is analogous to mutational meltdown in bacteria [31] or error catastrophe in viruses [32].

The concept of genomic convergence crystallized from observations that while new-born tumors may appear to hoard high levels of ITH, many later stage tumors are relatively less diverse [14, 33]. The picture that emerges then suggests that following an initial burst in diversity, war ensues among the subclones, leading to the extinction and death of “weaker” clones while only the superior clones or superiorly equipped alliances of clones—the powerful, well-adapted antiheroes with migratory and emigratory tendencies—prevail [33]. The consequent decline in diversity is probably accompanied by a significant decrease in CA. CA however is not completely eliminated, perhaps because extra centrosomes could aid in metastatic dissemination or in the switch back to a growth agenda once the migratory tumor cells have seeded in secondary locations.

And so the many-headed monster we started with, constantly shuffling genes to find the right combination to gain critical mass, now finds the right combination to not only fight the opposing army, but to create a small but powerful battalion of its own—metastatic tumors that will spread cancer's presence and bring the patient down once and for all. The rapid burst of increasing genetic diversity at the start gives way to very low mitotic propensity as a smaller number of well-selected clones that focus on migration and invasion rather than growth.

The above-described linear progression scheme places the expansionist pioneers (i.e., the metastatic founder cells) near the end of primary tumor development. As with everything else about the inventive, slippery beast that cancer clearly is, the reality is more complex and nuanced. It turns out that dissemination is often an early event in the progression of several, if not all, cancers [34]. The parallel progression model was postulated based on studies on doubling times in metastatic tumors that concluded that metastases were often initiated well before any symptoms appeared or the primary tumor was detected [35, 36]. So, when does cancer truly initiate his metastatic agenda? The answer perhaps lies, yet again, in ITH, owing to which each of cancer's clones switches to a migration-focused agenda at its own pace, functioning as a mini primary tumor of sorts. It is therefore not inconceivable that some of cancer's more ambitious legionnaires may embark upon their journeys, either alone or with a band of loyal followers and accomplices (some of whom may even be misguided host cells), very early in the lifetime of the disease, either finding temporary shelters to lie safe and further evolve independently, or rapidly colonizing new sites even when the “primary tumor” has barely found its footing [34, 37].

7 Scene six

7.1 The journey shapes the villain: centrosome amplification and mitotic propensity determine metastatic success

When exactly cancer shifts his attention from division and diversification, to dissemination, is a question of immense clinical relevance. It is frequently observed that despite complete resection of their primary tumor, patients with no overt metastases (i.e., M0 stage) at time of surgery also may eventually develop metastases; this observation suggests that the seed cells of distant metastases may have spread before the surgery or even before first diagnosis [38]. Intriguingly, these disseminated tumor cells, detected in organs such as bone marrow, often appear karyotypically normal and do not display signs of telomeric crisis or aneuploidy

(although some loss of heterozygosity is observed); these data repudiate the notion that acquisition of CIN necessarily occurs before the invasive stage of local progression [39]. One plausible explanation is that within a heterogeneous tumor, genetic, or epigenetic activation of migratory and invasive behavior via the right combination of early oncogenic alterations may, on occasion, even precede a dysregulation of cellular proliferation or induction of centrosome amplification. Moreover, tumor cell dissemination is the outcome of an interactive dialog between the neoplastic cells and the host cells that surround them and currently, not much is understood about what heterotypic signals or traits of the stromal microenvironment may facilitate local “leakage” of tumor cells early in the tumor's life. Regardless of what permits early dissemination, it is likely that the disseminated cells may not yet have acquired (at the point of exit from the primary site) the full array of traits needed for successful extravasation and colonization to form overt metastases. The further, primary tumor-independent genetic progression of early-disseminated M0 cells likely occurs via accrual of genetic and epigenetic changes and selection over a time period of “tumor dormancy” [40]. Clearly, this process of clonal evolution and expansion would depend upon both the acquisition of CIN as well as active mitoses, with the risk of overt metastases increasing with increased CA and faster cell cycling kinetics. In fact, a successful metastatic cell has been likened to a decathlon champion, who must be proficient in all ten events, not just a few, to be successful [41]. If early-disseminated tumor cells are out of the active growth-and-division cycles and thus are truly dormant, it is unlikely that they will ever be able to attain the multiple proficiencies required for successful escape from host defenses, adaptation to unfamiliar tissue microenvironments and colonization. Instead, acquisition of these multiple proficiencies is likely to depend upon active cell cycling and the continual reshuffling of tumor cell genomes via CIN, regardless of whether these steps occur within the confines of an advancing primary tumor or at an “intermediate” ectopic location [42]. So, cancer's renegade warriors may either train in-house or may go into hiding in clandestine training camps to learn and master new skills and emerge at a later time, more malevolent and better armed than before. By developing fine-grained portraits of tumors as a plurality of entities each with their unique life stories, our understanding of the covert and overt enemies we battle, begins to awaken.

8 Act two: putting Cancer's guns to its own head(s)

8.1 Scene one

8.1.1 Finding the guns that betray Cancer's dangerous intent: ITH and CIN are robust beacons of risk—Currently, clinicians rely on clinicopathological factors to inform them about the level of risk posed by cancer, to stratify patient populations and decide on appropriate therapy. In effect, this represents an attempt to profile the perversity that cancer is based on a snapshot of features that he revealed about himself at a certain point in time. But given that cancer is an entire army, with a multitude of faces and vile attributes, the clinician's image of his foe is inevitably incomplete, mutable, and unreliable. It is no wonder that current prognosticators have not achieved sufficient accuracy in predicting cancer's “risk level”. Hence, there exists an immense need to identify, develop, and clinically validate new biomarkers that reveal more about cancer's devastating battle

plans, his ammunition, his penchant for destruction, and his legionnaires' traits, if we are to have any hope of preempting his moves and defeating his forces.

One approach to better risk modeling has involved development of methods that assess the extent of ITH. Key advances in NGS have allowed us to appreciate the extent of ITH in hematologic or epithelial tumors, to help predict genomic divergence and convergence, and also to understand why treatments are failing [1]. An exome sequencing study in chronic lymphocytic leukemia found correlation not only between increased ITH and clinical progression, but also that chemotherapeutic interventions themselves acted as a driver for clonal evolution within the malignant cell population [43]. Another whole-genome sequencing study found that some dominant clones in acute myeloid leukemias acquired mutations at the point of relapse conferring resistance to chemotherapy; in majority of cases however, relapse was brought about by resistance mutations in a minor clone [44]. Unfortunately, NGS is labor intensive, expensive, and requires specialized expertise not only to perform but also to analyze the vast quantities of data generated. Though these sophisticated gene expression profiling techniques are useful and can yield much data, it is naïve to suppose that all these data are clinically actionable or that cancer can be defeated solely by targeting the dominant clones [1]. Moreover, analysis of a few of the most abundant cell types may lead clinicians to underestimate the tumor as a whole, and its future [1]. Clearly the very prevalence of extensive ITH in cancer questions the strategy of focusing on contributions of individual gene mutations or specific genetic aberrations to disease progression.

Recently, Almendro and colleagues published another method, based on immunofluorescence *in situ* hybridization (iFISH), to analyze cellular genotypes and phenotypes at the single-cell level. This study confirmed that ITH is a common, inherent feature of the tumor types studied, and that the extent of genetic diversity was similar regardless of the chromosomal region analyzed [45]. However, determination of the Shannon diversity index using iFISH is time-consuming, cost-prohibitive, and not clinically-facile. Other groups have employed gene expression-based analyses to quantify CIN, a key driver of diversity, to derive a biomarker for cancer state and disease progression. Carter and colleagues have developed a sophisticated computational method to characterize aneuploidy in tumor samples based on coordinated aberrations in expression of genes localized to each chromosomal region. The CIN25 signature showed good prognostic value in six different cancer types [46]. However, measuring CIN in this way is associated with all the drawbacks that accompany gene-expression-based methods including high cost, impractical turnaround times, and requirement for specialized labor. Clearly, clinically-facile methods to objectively and accurately measure ITH and CIN are yet to be developed, so it will be a while before their prognostic potential can be fully harnessed.

9 Scene two

9.1 Retooling our war against Cancer: Centrosome amplification and mitotic propensity are clinically facile biomarkers of risk

Cancer's ever-changing personality and dynamically evolving agenda mean there is an urgent need to develop novel biomarkers and combinatorial therapies; with cancer, one size

clearly does not fit all, all the time. Rather than attempting to resist the “attack of the clones” and finding ways to kill off highly evolved malignant cells, perhaps it is worth identifying tractable therapeutic targets that may limit the onset and propagation of ITH; silencing or slowing the machinery at the heart of cancer's shape-shifting personality, and doing this as early as possible? Approaches such as the use of centrosome declustering agents that capitalize on the vulnerability conferred by extra centrosomes on cancer cell targets are consonant with this idea. The mitotic propensity of tumors is another potential poor prognosis biomarker; frequent, error-prone mitoses are essential for CA to drive CIN and production of ITH. One could certainly explore the quantitative evaluation of CA and mitotic propensity as robust measures of risk, since the extent of CA and the inherent mitotic propensity of tumors play a key, determining role in the level of ITH the tumor attains (Fig. 1).

Indeed, extent of CA in tumor samples can be readily quantified through immunostaining of centrosomal markers such as γ -tubulin. CA has been in the past correlated to indices of increasing tumor aggressiveness, including tumor size, grade, metastasis, and recurrence in several cancer types [19,47–49]. Thus, measures of CA such as inappropriate centrosome number, aberrant shape, and function may allow prediction of tumor progression and clinical outcome. Routine profiling of centrosomal status may even identify patients who might be suitable candidates for centrosome-targeted therapies that throw a wrench in the works and could stall or reverse tumor progression. Similarly, new measures of the mitotic propensity inherent in tumors need to be developed by integrating mitotic and proliferation indices of tissue samples. Furthermore, the number of mitoses and proliferative cells can be easily counted, facilitating the establishment of ranges of mitotic propensity having prognostic relevance. Over time, with enough data, mitotic propensity could, like centrosome amplification, become a continuous variable that could be built into the next generation of prognostic models. We also foresee that these biomarkers could be critical in establishing relevant stratification criteria in the design of clinical trials and would thus become vital cogs integral to the machine of personalized cancer therapy.

If not representative of the whole cancer, targeted, personalized therapies with a singular mode of action will rarely be effective at conquering the many-headed monster [50]; in fact, destroying one type of cancerous clone may inadvertently change the landscape [51] and provide selection pressures for a different clonal type to dominate. Tumors constantly change in terms of the extent of ITH over time, in space, and in response to diverse factors within the tumor's microenvironment [51]. This, in turn, leads to many more unanswered questions: how many tumor samples are adequate to completely capture the spatial, mutational landscape of a given tumor? How frequently must samples be taken, in light of cancer's propensity to continually evolve new genetic diversity? Oncology units are increasingly adopting strategies to perform repeat biopsies of metastatic sites on disease progression as standard of care, but how should this be managed? Additionally, the timing of antimitotic therapy must be optimized—should it be applied in the early stages of tumor progression, or later; is antimitotic therapy useless when the tumor's agenda has moved away from mitosis towards metastasis? Clearly, we need to develop theoretical frameworks of the endless tools and strategies cancer deploys in his attack in order to generate relevant

testable hypotheses to enable development of novel biomarkers and therapies and the design of clinical trials.

Given that cancer's edge comes from complexity, overcoming the layered challenges posed by ITH is going to require an extraordinary vision and resolve and the willingness to embrace paradigm-shifting approaches. While the parallel progression model questions the validity of research models and clinical approaches that are solely based on biomarker assessment in primary tumors, it draws attention to the fact that diagnostic pathology needs to perhaps expand its scope to include biomarker analysis of systemic cancer. Although primary tumor profiling might yield reliable prognosis, predicting therapeutic responses will require the biomarker profiling of metastatic tumors which may be significantly different from the primary tumor. Parallel progression models also spotlight the need to center our efforts on searching for initiating and early predisposing alterations such as those that precipitate CA and derail proliferation, because these changes are more likely to be shared among primary and metastatic tumor cells and might influence the mutational and epigenetic spectra of subsequent changes. The practice of oncology needs to acknowledge that cancer is always a systemic disease because it is a readout for an imbalance between tumorigenesis and immune control of transformed cells. Therapies need to not only target the mechanisms that undergird tumor progression but also need to rope in immunotherapy modalities that could aid in tipping the scales in favor of tumor elimination or at the very least, maintenance of stable disease. In line with Nowell's assertion that "more research should be directed towards understanding and controlling the evolutionary process in tumors before it reaches the late stage seen in clinical cancer," [5] we propose that centrosome amplification and high mitotic propensity, which occur early in cancer's journey from normal cell to evil genius, are important potential prognostic biomarkers worthy of exploration. These biomarkers, that can be readily assessed by time-tested classical immunohistochemical methods, may affirm that old is indeed gold.

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Fig. 1.

Extent of centrosome amplification and inherent mitotic propensity determine the rate at which intratumoral heterogeneity (ITH) is generated. This schematic describes how a tumor cell population (with different degrees of centrosome amplification and mitotic propensity) in the vicinity of a blood vessel evolves over time. This tumor cell population could represent either an entire primary tumor, an individual clone within a primary tumor, or a metastatic tumor evolving in parallel with a primary tumor in another location. Four scenarios are illustrated for this population which is at a very early stage in its lifetime: Rows 1 and 2—evolution of ITH when two cell populations with similarly high mitotic propensity start off with either low or high levels of centrosome amplification, respectively. Rows 3 and 4—evolution of ITH when two cell populations with similarly low mitotic propensity start off with either low or high levels of centrosome amplification, respectively. Each row has four panels depicting sequential snapshots of tumor population over time. ITH level of the tumor population is represented by the histogram in the background, the color of the histogram representing the level/degree of ITH at a particular time/stage in tumor evolution. The height of the histogram depicts the maximum level of ITH attained in each of the four scenarios. Variety of clones produced is represented by the number of differently colored cells. The rate of ITH is depicted by the variety of clones produced, tumor size, and time it took the tumor to reach ITH peak (bright red part of the graph in the background)

which demarcates the switch in tumor agenda from mitosis to metastasis. Metastasis (if any) is depicted by *black arrows* pointing towards the blood vessel in the final panel in each row. Highest risk of metastasis occurs when both centrosome amplification levels and inherent mitotic propensity are high (Row 2).

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