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Turning the headlights on novel cancer biomarkers: Inspection of mechanics underlying intratumor heterogeneity

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Abstract

Although the existence of intratumoral heterogeneity (ITH) in the expression of common biomarkers has been described by pathologists since the late 1890s, we have only recently begun to fathom the staggering extent and near ubiquity of this phenomenon. From the tumor's perspective, ITH provides a stabilizing diversity that allows for the evolution of aggressive cancer phenotypes. As the weight of the evidence correlating ITH to poor prognosis burgeons, it has become increasingly important to determine the mechanisms by which a tumor acquires ITH, find clinically-adaptable means to quantify ITH and design strategies to deal with the numerous profound clinical ramifications that ITH forces upon us. Elucidation of the drivers of ITH could enable development of novel biomarkers whose interrogation might permit quantitative evaluation of the ITH inherent in a tumor in order to predict the poor prognosis risk associated with that tumor. This review proposes centrosome amplification (CA), aided and abetted by centrosome clustering mechanisms, as a critical driver of chromosomal instability (CIN) that makes a key contribution to ITH generation. Herein we also evaluate how a tumor's inherent mitotic propensity, which reflects the cell cycling kinetics within the tumor's proliferative cells, functions as the indispensable engine underpinning CIN, and determines the rate of CIN. We thus expound how the forces of centrosome amplification and mitotic propensity collaborate to sculpt the genetic landscape of a tumor and spawn extensive subclonal diversity. As such, centrosome amplification and mitotic propensity profiles could serve as clinically facile and powerful prognostic biomarkers that would enable more accurate risk segmentation of patients and design of individualized therapies.

Keywords

Intratumoral heterogeneity; Centrosomes; Chromosomal instability; Mitotic propensity; Biomarker

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1. Intratumoral heterogeneity: a tumor's much-desired destination

The last few decades of cancer research have yielded irrefutable evidence regarding the presence and strong clinical implications of interpatient and inpatient tumor heterogeneity (Almendro et al., 2014a; Bedard et al., 2013; Navin et al., 2010; Yap et al., 2012).

Theoretically tumors arise from a single transformed cell; however by the time of diagnosis, several genetically distinct populations of cells can be detected. This diversity within a tumor is referred to as intratumoral heterogeneity (ITH) (Maley et al., 2006; Park et al., 2010; Ye et al., 2009). Bewildering amounts of spatial and temporal ITH are now known to be present in several cancer types. In an analysis of 100 breast cancer genomes (Stephens et al., 2012), driver mutations were found in 40 different cancer genes in 73 different combinations. While 28% of these tumors had a single-driver mutation, the remainder had multiple alterations, with some tumors presenting with as many six driver mutations. Six driver mutations have never been intentionally targeted by clinicians at one time; this fact is certainly a wake-up call in terms of what a prohibitive challenge ITH poses for cancer treatment, especially when such treatment needs to be personalized for optimal outcomes. So profound is ITH in fact, that another breast cancer study in which single nuclei genome sequencing of 50 tumor cells was carried out, concluded very alarmingly, that “no two cancer cells within the same cancer have the same genome” (Wang et al., 2014). Moreover, ITH bedevils every stratum of the disease; within the primary tumor, between the primary and the metastatic lesion, and even between different metastases. Traditionally, clinical guidelines have focused on maximizing the patient populations that benefit from targeted therapies. In the face of incontrovertible evidence related to ITH, testing guidelines have been compelled to accommodate ITH. The 2013 ASCO/CAP guideline for breast cancer indicates that a tumor is HER2-positive if more than 10% of cells overexpress HER2. Additionally, the guideline states that heterogeneity should be reported (Wolff et al., 2013).

In recent years, viewing a tumor through the clarifying lens of ecological concepts has aided cancer biologists in developing frameworks for analyzing and understanding the dynamic population interactions that occur as ITH is generated (Cleary et al., 2014; Crespi and Summers, 2005); by focusing on “societal” relationships among cancer subpopulations, a better understanding of how these subpopulations can reciprocally influence each other's growth rate, metastasis, immune sensitivity, and therapeutic responses is gradually emerging. Among the fundamental interactions common to both diverse clonal subpopulations and species in an ecosystem are competition and cooperation (Fig. 1A). Levels of competition and cooperation depend on a wide variety of factors including varying tumor microenvironments, genetic, epigenetic and proteomic differences between neighboring cells, as well as availability of resources and the fitness of those cells in the niches present. These complex features of ITH provide important insights into therapeutic challenges frequently encountered in the clinic particularly when clinicians are confronted with aggressive tumor phenotypes.

What is the significance of considering a tumor as a diverse ecosystem? Diversity has been known to stabilize an ecosystem in the face of selective pressures and environmental changes. Diverse ecosystems are more productive, filling available niches and using resources more effectively (Cardinale, 2011; Hautier et al., 2014; Hector, 2011). Stability, in

an ecological context, entails remaining productive (*i.e.* producing biomass) despite challenges to the ecosystem (Bezemer and van der Putten, 2007). For example, if a prairie undergoes a drought, there will be a decline in the number of grass species that cannot withstand excessively dry conditions. However, those grasses that can withstand the harsh conditions will be more productive, resulting in very little change in overall ecosystem productivity and the continued efficient use of available resources. To draw a parallel with tumor biology, a particular species in an ecosystem would be analogous to a particular tumor cell clone, and different species would be equivalent to cancer cell subclones that have diverged genetically from their parents. A resistant tumor displays growth and stability, even in harsh conditions such as chemotherapy or radiation treatment, similar to a diverse prairie ecosystem.

In addition to facilitating the development of therapeutic resistance, diversity could also provide a multitude of benefits that might aid in metastasis, a key phenomenon underlying poor prognosis. While the prairie grass example earlier highlights adaptability of an ecosystem where the players involved are static, diversity within mobile populations can lead to successful migration out of crowded areas into new niches. These new environments can be physically distant or proximal with differing resources and conditions. It has long been posited that the process of metastasis exerts considerable selective pressure on the migrating tumor cells (Fidler and Hart, 1982). The larger the pool of diverse clones metastasizing from a tumor, the more likely one will not only survive the migration process but also be better adapted to a new site. As ITH develops, some clones will be less suited for the current environment but would be capable surviving the voyage to, and settlement of, a more suitable secondary site. In essence, high levels of ITH would allow for therapeutic resistance and metastasis, both important phenotypes associated with advanced and aggressive cancers.

The key population dynamics of cooperation and competition have been implicated in instances of cancer relapse and highlight new challenges for designing suitable treatment regimens and modalities. As chemotherapy targets a dominant clone population, the competing smaller subpopulations expand to fill the vacated niche (Burrell and Swanton, 2014b; Landau et al., 2014). These subclones could contain more aggressive driver mutations than the previous dominant clone. It is extremely challenging to detect all the tumor cell subpopulations that are present before the commencement of any particular treatment. Even more difficult is predicting the final impact of therapy on tumor progression, by elucidating how the treatment would impact each of these populations as well as the dynamic interactions among them (Fig. 1B). While a large body of data supports the presence of competing subpopulations of tumor cells within tumors, in 2011, Anderson et. al. uncovered evidence that multiple subclones played a role in relapse; suggesting cooperation among clones. It is now believed that cooperation between clones likely contributes to relapse in cases of acute lymphoblastic leukemia (Anderson et al., 2011; Burrell and Swanton 2014b). Cleary et al. likewise uncovered the mechanistic details underlying an essential cooperative interdependence between two distinct subpopulations within breast cancer tumor model (Cleary et al., 2014). Furthermore, Polyak et al. found that minor populations can meaningfully contribute to tumor growth and stabilize it against

collapse (Polyak and Marusyk, 2014). Therefore, ITH, while providing insight into the mysteries of relapse, also increases the complexities to consider when planning treatments. ITH poses challenges to both clinicians and drug developers to detect low level clones, predict tumor evolution, develop drugs to target specific clones and evaluate effective, yet non-toxic combinatorial regimens to combat ITH. Given that there are countless trajectories of tumor evolution to map (Fig. 1B) in the hope of leading tumors to “evolutionary dead-ends” (Burrell and Swanton, 2014a), and trying to fight an army of superiorly evolved clones is unlikely to meet with significant success, it might be a more prudent strategy to target the vehicle that drives a tumor to its desirable destination of ITH.

As strong correlations between ITH and aggressive disease course emerge (Maley et al., 2006; Park et al., 2010), it becomes crucial to thoroughly study how ITH varies between primary and metastatic sites, at different stages of disease progression, and during different treatments. However, in order to correlate the risk contribution of ITH to a tumor, powerful and clinically-facile methods to quantify ITH need to be developed and standardized. Because ITH is likely to fluctuate depending on site, stage and treatment status, the propensity of a tumor to develop ITH will likely factor strongly into determining risk. As such, the drivers of ITH are poised to emerge not only as indicators of diversity potential and “risk of aggressiveness” but also as critical therapeutic targets. Once a tumor, despite its other attributes, arrives at the ideal level of ITH, it is rendered less sensitive to therapy and more likely to metastasize. The questions to ask now are: what leads a tumor to ITH? And will targeting the drivers of ITH improve patient outcome by preventing a tumor from growing and evolving to become resistant or metastasize? If so, the daunting task of developing biomarkers to measure ITH levels in tumors will become a critical prelude to the rational design of treatments.

2. Chromosomal instability: “CIN”ful vehicle takes a tumor to its destination in style

Numerous causes of genetic variation can contribute to a tumor’s population diversity; however a strong correlation between chromosomal instability (CIN) and poor prognosis, as well as CIN and ITH has been established (Brinkley, 2001; D’Assoro et al., 2002; Pihan et al., 2003) CIN is broadly defined as the rate of change in karyotype and often leads to aneuploidy. It is well established that aneuploidy itself can initiate tumorigenesis (Duesberg et al., 2006; Weaver et al., 2007), however, aneuploidy in healthy cells like hepatocytes can be stable, while CIN, as its name implies, is dynamic and leads to chromosomal missegregations and karyotypic alterations occurring on an ongoing basis (Heng et al., 2013). This instability leads not only to tumorigenesis (Burrell and Swanton, 2014a; Lee and Swanton, 2012) but enables the tumor to continually evolve, developing resistance to therapies and metastatic phenotypes. The striking correlation between ITH, CIN and poor prognosis is intriguing and leads us to hypothesize that ITH acts as an ideal destination for a tumor. A pool of diversely malignant cells is better equipped to overcome a variety of external pressures like therapeutic interventions and internal pressures such as metastasis. As such, CIN is a powerful vehicle carrying the tumor to its desired destination. Much like other vehicles, this ‘CIN vehicle’ is not on a single track and could lead to ITH via a

plethora of unique routes, directed by various drivers. However, the rate of tumor progression can only be as efficient as its engine. In a later section, we describe the engine that powers the “CIN”ful vehicle of a tumor.

2.1. Keeping CIN in check: speeding increases fatal accidents and arrests

Just like the complex relationship between speed and the number of accidents, the relationship between CIN and prognosis is not a straightforward one. While CIN, similarly to ITH, is generally correlated with poor prognosis, some evidence indicates that very high levels of CIN are associated with improved patient outcome (Swanton, 2012). This is consistent with the concept that extreme genetic instability leads to more instances of mitotic arrest and lethal mutations as fewer daughter cells receive a functional genome. It then follows that effective and persistent CIN must allow tumors to speed toward ITH while at the same time evading the consequences of high-grade aneuploidy, mitotic arrest and accumulation of lethal mutations. So what are the mechanisms underpinning CIN? And do they differ in their ability to evade these negative consequences? Is there a driver who knows that speeding too fast increases the chance for fatal accidents and arrests?

3. The drivers of CIN

3.1. Mutations in spindle assembly checkpoint genes: a neglectful hurried driver

The spindle assembly checkpoint (SAC) is a mechanism that senses both microtubule (MT) attachment to kinetochores and tension between sister chromatids (Lopes and Sunkel, 2003) in order to communicate if they are improperly completed and prevent the onset of anaphase until errors are corrected (Tan et al., 2005). The elucidation of the molecular mechanisms behind this checkpoint has led investigators to question whether SAC gene mutations underlie CIN associated tumorigenesis (Lopes and Sunkel, 2003). Aside from one study that indicated a direct link from mutations in the human SAC gene Bub1 to a colorectal cancer (Cahill et al., 1998; Lopes and Sunkel, 2003) numerous studies have shown that spindle checkpoint mutations are rarely coupled with tumor development (Lopes and Sunkel, 2003). Although there is a correlation between mitotic spindle checkpoint malfunction and CIN, conclusive evidence showing that compromised SAC function is a major contributor to ongoing CIN is still lacking.

Like a negligent rushed driver, the pre-transformed cell disables or ignores “check engine” warnings of the cell cycle and speeds ahead. When alone and uncompensated, mutations in SAC genes are likely to result in cancer cells proceeding with the cell cycle despite the presence of errors that would normally halt their progression; the result is that these errors often result in lethality or nonviable daughter cells. Therefore, SAC mutations, while contributing on occasion to ongoing CIN, are more likely to lead to unchecked cancer cell replication, once transformation has occurred and a tumor has begun to develop.

3.2. Improper kinetochore attachment: which pedal's the brake?

Even with a functional SAC, certain improper spindle-kinetochore attachments can fulfill the requirements of attachment and tension to pass the SAC checkpoint ultimately contributing to a CIN phenotype (Thompson and Compton, 2008). The attachment of MT to

kinetochores is a dynamic process and the binding of one kinetochore to more than one pole (merotelically) is common in early mitosis (Bakhoum et al., 2009; Cimini, 2008; Zhai et al., 1995). However, if the rate of merotelically surpasses the correction rate, the cell may proceed through mitosis with increased incidences of lagging chromosomes, a characteristic linked with CIN (Bakhoum et al., 2009). There are many MT-associated proteins tied to the development and maintenance of correct spindle kinetochore attachments during mitosis. For example, hepatoma up-regulated protein, also known as HURP, is thought to stabilize the spindle at the metaphase plate. HURP null mutants have higher rates of lagging chromosomes (Breuer et al., 2010). On the other hand, excessively stable kinetochore MT bonds were shown to decrease the ability of cells to correct merotelically, leading to lagging chromosomes (Bakhoum et al., 2009). Interestingly, in those same experiments, certain cell lines (RPE1 and HCT116) remained relatively diploid despite rates of lagging chromosomes similar to aneuploid CIN tumor cells (Bakhoum et al., 2009). These data suggest that merotelically alone was insufficient to confer the CIN phenotype on cell lines that (i) were not initially genetically unstable, and (ii) did not harbor supernumerary centrosomes (Holland et al., 2012; Lentini et al., 2007). To summarize, while a functional imbalance of spindle checkpoint and/or kinetochore attachment proteins could cause a CIN phenotype on occasion, it is unclear whether these imbalances would persist and be able to continually contribute to low level chromosomal mis-segregation. Additionally, these drivers seem ill-equipped to avoid the growth and proliferative disadvantages resulting from gross levels of aneuploidy. The multiple missegregation events that would occur by merotelically would need to be paired with a compensatory mechanism to allow for successful division of resulting aneuploid cells (Thompson and Compton, 2008). In sum, like an inexperienced driver, cells with a propensity for improper kinetochore attachments confuse the pedals of the car, the brake, the accelerator, and the clutch. A vehicle that is driven in such a manner, speeding and stalling, is at best wasteful and at worst causes fatal accidents and arrests, either of which is prohibitive for normal steady headway towards a destination.

4. Centrosome amplification: a key driver of CIN that uses illegal radar detection

Centrosome amplification (CA) is the increase in size and/or number of centrosomes that results from dysregulation of the centrosome duplication pathway, *de novo* centrosome biogenesis and/or failure of cytokinesis (Nigg, 2006). Centrosomes are key for the nucleation and organization of microtubules and play critical roles in cell polarity, assembly of the mitotic spindle and in ensuring equal partitioning of the genome between daughter cells through each round of cell division (Khodjakov, 2001; Ogden et al., 2012). Therefore a functional increase in centrosomes can theoretically affect not only mitosis but also all of the processes involving microtubules from cell polarity maintenance and cell migration to cell cycle progression (D'Assoro et al., 2002; Ogden et al., 2013). We will focus mainly on centrosome amplification's roles in the cell cycle and mitosis as those contribute most to its role in CIN. In the following sections, we will discuss how amplified centrosomes behave like unsafe drivers who get away with speeding due to use of illegal radar detectors that help them elude arrest by law enforcement; amplified centrosomes cleverly enlist the support of

mechanisms that allow the cell to avoid potentially lethal multipolar mitoses and maintain an optimally low level of CIN through frequent merotelic and occasional chromothripsis.

4.1. Centrosome amplification: inherent driving talent or learned skills?

In the process of reevaluating Boveri's 100 year old observations that multiple centrosomes are often seen in tumors, centrosomes have been investigated for their role in aneuploidy, tumorigenesis, and metastasis (Heim, 2014). CA, which occurs widely in both solid and hematological malignancies, has been shown to positively correlate with both tumor grade and malignancy (Giehl et al., 2005; Lee and Swanton, 2012; Lingle et al., 1998) although more rigorous and standardized quantitation needs to be carried out. Amplified centrosomes have been observed in breast, prostate, pancreatic, colon and ovarian cancers as well as premalignant lesions (Chan, 2011; Godinho and Pellman, 2014; Pihan et al., 2003). In previous studies using the HPV E7 oncoprotein to disrupt the centrosome duplication cycle, an increase in centrosomes was observed before the development of tumors (D'Assoro et al., 2002; Duensing et al., 2000). Together, these data suggest that CA occurs early in, and potentially even before, tumorigenesis, and therefore could be a critical and powerful driver of tumor progression.

But what endows tumors with this "excess baggage" of too many centrosomes? The centrosome duplication pathway is normally very tightly regulated. Many proteins including separase, Plk1, ORC1 and MCM5 are implicated in licensing the duplication of centrosomes in strict synchrony with specific events of the cell cycle (Ogden et al., 2012). Considering the need to coordinate centrosome duplication with other cell cycle processes, it should come as no surprise that some cell cycle regulatory proteins such as cyclin dependent kinases (Cdks) also control the centrosome duplication. Due to both their redundancy and specificity, the role of Cdks in centrosome replication has been challenging to decipher (Harrison et al., 2011). It has been suggested that the dysregulation of the cell cycle by tumor suppressors and oncogenes can lead to a loss of control of the centrosome cycle (Fukasawa, 2007, 2008; Harrison et al., 2011). The intimacy of these two cycles begs the question, which is cause or consequence? Is it possible that deregulation of centrosome duplication causes the genetic alterations in tumor suppressor genes and oncogenes? Some proteins associated with both centrosome homeostasis and checkpoint control such as p53 (Morris et al., 2000), CDC2/Cdk1 (Pockwinse et al., 1997), and BRCA1 (Hsu and White, 1998; Xu et al., 1999) are localized at the centrosome, which suggests that perturbations in centrosome balance could further alter checkpoint and centrosome duplication control (D'Assoro et al., 2002). Each of these proteins is important to maintaining genomic stability: BRCA1 contributes to proper spindle formation (Starita et al., 2004), Cdk1 promotes proper kinetochore attachment and tension (Choi and McCollum, 2012), and loss of p53 function has been shown to induce centrosome amplification. Imbalance of these proteins due to an imbalance of centrosomes then describes a powerful mechanism by which CA drives CIN.

In addition to causing imbalances in proteins critical for proper cell cycle/checkpoint maintenance, amplified centrosomes could have implications for both merotelic and cohesion defects both of which are causes of aneuploidy and CIN. These effects stem from centrosomes' effects on the microtubule (MT) nucleation. Increased rates of merotelic

attachments could be due to the increase in overall MT nucleation sites. For example, if the ratio of centrosome-anchored MTs available to attach to a kinetochore is higher than the number of kinetochores, the probability of making unfavorable attachments increases. This likelihood is further increased when considering two facts: (i) kinetochores preferentially attach to MTs they are facing (Bakhoum et al., 2009; Nicklas and Ward, 1994), and (ii) cells with supernumerary centrosomes often pass through a transient multipolar spindle state prior to centrosome clustering to assemble a pseudo-bipolar mitotic spindle (Ogden et al., 2012). During this phase, MTs emanating from different centrosomes can attach to a single kinetochore; eventually, such mis-attached kinetochores could end up at the same pole when centrosome clustering occurs (Ganem et al., 2009; Ogden et al., 2013; Silkworth et al., 2009). In this way CA is a potent driver of CIN through merotely. CA can like-wise drive sister chromatid cohesion/separation defects. Unequal total MT nucleation at each pole during mitosis could cause unequal separation. Referred to as cohesion defects, these are thought of as faults in the cohesion of the sister chromatids, however, similar effects could theoretically be seen as a result of asymmetric nucleation capacity at each pole (Lingle et al., 2002; Ogden et al., 2013; Yamashita and Fuller, 2008). While merotely and sister chromatid cohesion defects have been implicated in chromosome missegregation, a likely culprit behind persistent low frequency perpetuation of these mechanisms is CA.

5. Centrosome clustering: directing centrosome amplification's driving power

In cells with extra centrosomes, spindle pole focusing of MTs relies on mechanisms that are critical for allowing cells to evade both spindle multipolarity and intolerable levels of chromosomal loss during mitosis by allowing the formation of a “pseudo-bipolar mitotic spindle” (Ogden et al., 2012). Thus far, a large number of factors including SAC proteins, actin, microtubules, and certain MT-binding proteins have been proven necessary for effective centrosome clustering during mitosis (Kwon et al., 2008; Ogden et al., 2012; Rebacz et al., 2007). Without centrosome clustering, CA's key to CIN success, cells with supernumerary centrosomes would be susceptible to multipolar spindle arrest and/or production of nonviable daughter cells from cell divisions that are accompanied by lethal levels of chromosome loss. Instead, clustering allows for persistence of low-grade chromosome missegregation that contributes steadily to ITH and tumor evolution. Interestingly, some MT-binding proteins that appear necessary for centrosome clustering also offer promising targets for therapeutics (Kwon et al., 2008; Ogden et al., 2012). One such protein, KIFC1, also known as HSET, is a minus-end MT motor protein that positions itself between microtubules and contributes to spindle pole focusing of MTs and centrosome clustering (Gordon et al., 2001; Ogden et al., 2012). Interestingly, HSET appears non-essential in healthy somatic cells (Godinho and Pellman, 2014; Mountain et al., 1999) but plays a vital role in maintaining the delicate balance between spindle pole separating forces and centrosome clustering forces mediated by a host of proteins like ninein, CLASP, Kid, CENP-E, and NuMA (Godinho and Pellman, 2014). In cells containing extra centrosomes, drugs that interfere with clustering mechanisms disrupt that balance and cause multipolar mitotic catastrophe (Pannu et al., 2014). Centrosome declustering drugs thus show great promise in specifically targeting cancer cells with extra centrosomes (Pannu et al., 2014). By

preventing these more adaptable cells from surviving, declustering drugs not only specifically target cancer cells with CA but also likely direct tumor cell populations to be less genetically malleable and prone to developing ITH.

5.1. CA and chromothripsis: using the express lane

Centrosome amplification's most efficient mechanism of large-scale karyotype reshuffling likely lies in its contribution to chromothripsis. This pulverization and subsequent repair of the chromosome is thought to occur both while chromosomes are condensed (*i.e.* during mitosis) and early in tumor development (Forment et al., 2012). Stress induced genome chaos, such as chromothripsis, has been linked to prompt surges in drug resistance (Heng et al., 2013; Zhang et al., 2013). While the exact cause of chromothripsis is not universally agreed upon, potential causes include radiation, escaped apoptosis, breakage–fusion–bridge cycles, and more popularly, micronuclei formation (Forment et al., 2012). Micronuclei have been shown to result from lagging chromosomes or pieces thereof that can become trapped within cleavage furrows during cytokinesis. DNA trapped in micronuclei may undergo replication out of synchrony with nuclear DNA, resulting in DNA damage and often extensive pulverization of the DNA in the micronucleus. Random rejoining of these fragmented chromosomal segments can produce chromosomes with thousands of rearrangements. These massively rearranged chromosomes may even get incorporated into the main cell nucleus during subsequent cell cycles where their persistence could lead to very significant changes in cellular karyotype and phenotype (Crasta et al., 2012; Forment et al., 2012). Supernumerary centrosomes increase the incidence of lagging chromosomes and micronuclei and as such, CA likely contributes to genomic instability through the mechanism of chromothripsis. The stress to chromosomes caused by merotelic attachments and/or centrosomal nucleation imbalances could also contribute mechanically to chromothripsis. This is a powerful mechanism but likely one that is present in the punctuated phase of the tumor evolutionary cycle thus providing a base of heterogeneity to the tumor.

In short, centrosome amplification is a powerful driver of CIN and thus accelerates emergence of ITH in tumors. CA has been documented in tumors from a variety of tissues, is associated with advanced tumor grade and malignancy, and is thought to occur early in tumorigenesis. It should be noted that progeny of tumor cells with amplified and clustered centrosomes also inherit excess centrosomes. Therefore the benefits of CA are inherited by progeny cells allowing CIN to be persistently generated.

5.2. CA drives CIN to ITH: a good engine and a smart driver will get you there

CIN is critically important because it results in ITH, which predisposes a tumor to metastasis and resistance, the key characteristics of an aggressive phenotype. Once a pool of genetically diverse clones has formed in a tumor, that diversity can self perpetuate as competition and cooperation play out in the tumor ecosystem. The more diverse this pool, the more likely a clone will be genetically equipped to handle the highly selective pressures needed to successfully metastasize and resist therapies. Drastically different cellular proteomes are needed in order for a clone to travel to and colonize a distant tissue. The clone must be able to proliferate free of its contacts with the tumor, to survive the conditions of the

lymphatic or circulatory systems, and to continue to evade the immune system. Once a tumor cell clone (or subclone) arrives at a new site, it must be able to adapt to potentially drastically different environments. Although the ability of groups of cells to metastasize together (Hart, 2009) is helpful, starting with a large pool of heterogeneous clones is critical for this process.

Similarly, diversity underlies the resistance potential of a tumor. Swanton's group has likened the tumor to a tree and treatments to the cutting of branches (Lee and Swanton, 2012) with increased diversity in a tumor being metaphorically equivalent to a tree bearing more branches. Should a treatment target a characteristic shared by a small branch of the tree, the tumor would likely survive. Importantly, if the cause of ITH, CA-driven CIN, is still present, then the tumor could repopulate with new clones theoretically both sensitive and resistant to the treatment (Bennett et al., 2004; Prosser et al., 2009). This is supported by studies that correlate CIN with drug resistance in both *in vitro* studies (Lee et al., 2011; McClelland et al., 2009) as well as through meta-analysis of clinical studies (Lee et al., 2011).

Centrosome amplification may thus employ chromothripsis, merotelly, and spindle checkpoint manipulations in order to persistently drive genetic instability toward ITH while using clustering mechanisms to navigate CIN around pitfalls.

6. Mitotic propensity: the powerful engine propelling the vehicle of CIN

It should be noted that the development of ITH requires cell division without which genetically distinct daughter "subclones" could not be generated. This explains the partial success of current cancer treatments that target mitotic cells; they also inadvertently slow the development of ITH. The turnover rate of proliferating cells in a tumor is herein referred to as mitotic propensity (MP). Tumors with a high propensity to undergo frequent and erroneous mitoses are likely to generate rampant CIN, especially if accompanied by CA. Thus, the amount of heterogeneity in a tumor is a product of the level of genetic instability (how much the genome can change per division) and the mitotic propensity (how often division takes place). The extent of ITH, and therefore the propensity for resistance and metastasis that develops, is dependent on both of these factors. For example the most extensive ITH would be the result of pervasive CA and high MP. From this ideal fortress, ITH, the tumor can both resist chemotherapeutic treatments and adapt to the highly variable conditions of metastasis. As both CA and MP work together to generate ITH and contribute to the risk (of poor clinical outcomes) associated with a tumor, ideally both factors would need to be measured to assess risk more accurately and stratify patients into prognostic subgroups.

7. Using ITH to predict risk: are we there yet?

If ITH is indeed the much-desired destination for a tumor and more ITH means poorer prognosis, then identifying how much ITH a tumor has garnered would have significant impact on prognostication and customization of therapy. As such ITH has been proposed as a powerful biomarker (Schwarz et al., 2014). Methods for quantifying diversity have been borrowed from ecology and adapted to the cancer field (Almendo et al., 2014b). While

distinct techniques have provided valuable evidence as to the presence of ITH and its implications for treatment (Almendo et al., 2014a; Ding et al., 2012; Lohr et al., 2014), they are yet to be adapted and sufficiently tested as an adequate readout of risk. Like-wise, measures of the “CIN”ful vehicle that drives a tumor toward ITH are proving inadequate and impractical as clinically applicable evaluations of ITH. The current challenges related to quantitative assessment of ITH, and CIN, will be briefly discussed later.

Next generation sequencing (NGS) is a powerful investigative tool that has provided evidence to explain some of the failures and successes of cancer therapy (Ding et al., 2012; Lohr et al., 2014). It is being used in “biomarker-driven” trial designs to account for heterogeneity (Catenacci, 2015). However, NGS is not widely being proposed as a way to quantify ITH as a biomarker itself, likely due to the great monetary and training costs needed to clinically implement it, lengthy turnaround times and the specialized expertise required to run NGS and interpret its results. Also, the data churned out by NGS are not all immediately actionable (Schwarz et al., 2014).

In order to quantify diversity and thus ITH, ecological diversity indices have been adapted to tumor biology. Specifically, the Shannon and Simpson diversity indices use estimations of the number and quantity of each identifiable subclone (which are analogous to ecological species) (Almendo et al., 2014b; Maley et al., 2006). Herein lie the devilish details of how and to what depth subclones are distinguished; some have used combinations of phenotypic and genetic characteristics such as surface protein markers and gene hybridization probes (such as those used in iFISH), respectively (Almendo et al., 2014b). Others have measured diversity using multiple genetic indicators such as loss of heterozygosity in microsatellites, mutations in specific genes of interest, and ploidy (Maley et al., 2006). In these cases, investigators identified distinct clones based on unique combinations of characteristics and estimated their relative abundance in order to quantify diversity. However, as Park et al. stated, ITH is difficult to confine as it encompasses many characteristics including, but not limited to, gene expression, epigenetic modifications, and mutations (Park et al., 2010). Should investigators pursue using ITH as a biomarker, standardizing how the “species” of tumor cells are distinguished will be both critical and immensely challenging. Of course, compounding these issues of estimating number and abundance of these “clone species” is the issue of tissue sampling limitations due to limited number of biopsies that tend to be performed on each patient. So while thus far, the diversity indices have been used primarily to quantify differences in diversity within individual sets of experiments, the constraints and practicality drawbacks make the standardization and implementation of quantified ITH as a biomarker, impracticable.

Most recently, a study using an innovative algorithm, MEDICC, estimated ITH using genome-wide single nucleotide polymorphism (SNP) array data from 14 ovarian cancer patients (Schwarz et al., 2015). This technique reconstructs phylogenetic lineages of tumor cells based on the genomic differences detected in SNPs and Copy Number Alterations (CNAs), and produces a heterogeneity measurement. The authors found that although ITH did not predict survival, it was consistent with their finding that resistant clones are present in a tumor pretreatment. Post treatment, these clones are likely selected for and undergo clonal expansion, which correlated inversely with survival (Schwarz et al., 2015). Such

studies are critical to elucidate the patterns in heterogeneity during disease progression and treatment to set the groundwork for more clinically applicable surrogate markers.

Another strategy to approximate ITH is to measure the speed of the vehicle of tumor evolution, CIN. Ye's group has found that nonclonal chromosomal aberrations (NCCA) are indicative of relative instability and are a more accurate measure of CIN than the previously stressed clonal chromosomal aberrations (CCA) (Heng et al., 2013). Essentially, CIN (measured by NCCA) marks the critical punctuated phase of tumor evolution in which the genome is often drastically rearranged. While this phase is accompanied by higher rates of cell death due to nonviable karyotype arrangements, it is a critical mechanism used for large evolutionary jumps (Heng et al., 2013). CCAs mark the stepwise phase of tumor evolution in which more stable populations mutate gradually and give rise to the collection of epigenetic and genetic signatures that have been so extensively probed. As fewer clones are sacrificed in the pursuit of perfection during this stable stage, reduced CIN is associated with increased growth (Heng et al., 2013). As a tumor cycles through these two stages, we can see that CIN and MP play vital roles in driving the development of ITH.

However, despite these more recent insights into accurately measuring CIN there are still many challenges. Large sample sizes, often needed for certain CIN measuring techniques like competitive gene hybridization (CGH) arrays, can be prohibitive. Unfortunately, high throughput methods like CGH are often not sensitive enough to detect cell-to-cell variation as these genomic arrays are based on "average population profiles." Additionally, the large number and diversity of aberrations make it a challenge to implement these procedures into routine patient tests (Heng et al., 2013). In order to achieve an accurate measure of CIN, these aberrations would optimally be measured over time; however, that, too, is often impractical. While some genetic profiles have been developed, for example the CIN25 signature, in an attempt to assess genomic instability and therefore aggressiveness potential (Blighe et al., 2014; Carter et al., 2006; Parast and Cai, 2013), these methods present many of the same drawbacks as those associated with CGH and are currently inapplicable to the clinic. Using interphase fluorescence *in situ* hybridization (iFISH) to measure CIN poses a different set of challenges. Although more technically facile, iFISH is labor intensive and would require extensive standardization as well as multiple biopsies to determine the rate of karyotype alteration. In conclusion, measuring ITH or CIN with the current methods is not conducive to translation to the clinic for routine use.

8. CA and mitotic propensity as prognostic biomarkers: the trails less traveled

Although we may be a far cry from implementing measures of ITH as a prognostic biomarker, quantitation of CA and MP, which are key drivers of ITH, may serve as robust and clinically-facile surrogate measures of risk, thus enabling more precise diagnoses, more accurate prediction of disease progression, patient response to specific therapies and disease outcomes. One reason is that CA acts as a critical driver of CIN, and MP is its vitally essential engine. In addition, CA has been recognized to correlate with tumor aggressiveness, as well as potentially having a causative role in transformation (Fukasawa et al., 1996; Michor et al., 2005; Ye et al., 2009). There are still several outstanding questions

concerning potential differences between structural and numerical centrosomal aberrations in terms of their distinct contributions to disease pathology that need to be investigated. Given that one would expect fluctuations in ITH during disease progression, corresponding trends in CA should be tracked and correlations between ITH and CA should be closely scrutinized to validate that (i) CA is indeed a robust surrogate of ITH, and (ii) incorporation of CA into the next generation of prognostic models indeed improves the accuracy of patient stratification and facilitates clinical decision-making for personalized therapy. Rigorous statistical analyses of correlations between clinicopathologic factors (such as tumor grade, stage, outcome) and CA, and the identification of cutoffs for determining risk categories would require analyses of centrosomal profiles of large cohorts of patient samples accompanied by good quality clinical outcome data. The key advantage of developing centrosomal status as a prognostic biomarker is ease of determination of CA using routine immunohistochemical staining of established centrosomal markers such as γ -tubulin. Additionally, measurements of CA and MP would avoid the need to qualify the relationships (competitive or otherwise) between clones present in the heterogeneous tumor. These surrogates of ITH would also be broadly applicable across a diverse range of cancer types.

Centrosome status alone, however, would not be a sufficient indicator of prognosis, as there are other elements that contribute to the efficiency of CA in producing CIN. For example, the abundance and proficiency of clustering mechanisms may vary. High levels of clustering machinery paired with increased centrosomes may lead to optimal levels of CIN and indicate poor prognosis while the same lower levels of clustering proteins in conjunction with increased centrosomes may correlate with improved patient survival. Mitotic propensity, or the rate of cell division among proliferative cells, also plays a role in a tumor's timely arrival to ITH. While mechanisms driving CIN may be supremely efficient, if they are coupled with a low mitotic propensity the tumor would be slow to develop ITH. Also, CA winds the tumor to ITH not necessarily taking the most traveled routes. Thus, determining the distance from ITH, or ability to arrive there, might require additional information about the fuel and efficiency rating of CIN. Additionally, the CIN vehicle needs enough fuel to reach its destination. If the pool of proliferative cells were construed as the fuel that enters the engine (mitotic propensity), it is amply clear that several factors would need to be considered in an integrated manner to derive a measure that closely mirrors the extent of ITH present in a tumor (Fig. 2). Importantly, most of these factors may be quantitated via routine immunohistochemistry; thus, it is likely that the most clinically-facile method of quantifying ITH may ultimately emerge from a model involving these hitherto overlooked risk-contributing factors. CA and MP therefore merit the opportunity to undergo rigorous clinical validation as ITH surrogates.

Implementation of next-generation risk models that sufficiently honor key risk contributors such as CA and MP could become the cornerstone of patient stratification in due course. In addition, measurement of CA and MP could permit convenient dynamic evaluation of ITH for evaluating efficacy of new treatment modalities and regimens. For instance, it has been demonstrated that chemotherapy may target more proliferative cell populations in a tumor, thereby selecting inadvertently for slower-growing cells that resist treatment. It has therefore been postulated that the administration of chemotherapeutic agents at lower doses and on a frequent schedule – Metronomic therapy – could in theory slow down such selection

processes and perhaps yield higher therapeutic efficacy (Montagna et al., 2014). Development of CA and MP as surrogate markers for ITH would allow correlations between progression of metronomic therapy and trends in ITH to be readily evaluated. Another experimental therapy, termed Adaptive therapy (Gatenby et al., 2009), has been proposed as a strategy that could maintain a stable population of “fitter” chemosensitive cells, which would keep the resistant cells (that face a fitness disadvantage due to their inherently higher energy demands) at a minimal fraction. It has been suggested that this approach could turn cancer into a manageable chronic condition, and that “stable disease” might be a better outcome in the long-term than trying to eradicate cancer completely. Robust surrogate measures of ITH such as MP and CA would go a long way in determining the clinical potential of these and other novel experimental approaches, combinatorial drug regimens and dosing schedules in the hope that they may yield new opportunities for therapy or provide greater efficacy compared to currently-used regimens. But perhaps the greatest clinical promise may lie in targeting the molecular mechanisms underlying CA and MP in order to incapacitate the mechanisms by which the tumor arrives at ITH. After all, no car can hit the road, let alone arrive at the destination, without its drivers. By modifying our perspective, focusing on the drivers and the practical implementation of suitable surrogates, it is possible that ITH, the clinical problem of alarming proportions, may itself become part of the solution.

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Fig. 1. Effect of treatment on population dynamics within heterogeneous tumors. (A) In heterogeneous tumor population dynamics of competition (top) and cooperation (bottom) are at work. (B) Should treatment differentially affect the distinct populations within a tumor, the outcomes become increasingly difficult to predict. Panels show the basic scenarios with the following stipulations: Red and Orange populations cooperate as do Blue and Green. However, Red/Orange compete with Blue/Green. Post treatment, a net decrease in tumor size could result from scenarios including treatment equally affecting all

populations in question and treatment mostly affecting more aggressive populations (Red/Orange) as shown in B, top panel. Note this does not preclude the evolution of additional clones (see Purple population). No net change in tumor size (B, middle panel) may result from increased sensitivity to treatment of either Orange (top middle panel) or Red (bottom middle panel). Net increase in tumor size (bottom panel) could result from more sensitivity to treatment of the Green (top of bottom panel) or Blue (bottom of bottom panel) populations.

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Fig. 2.

On the Road to Intratumoral Heterogeneity: Centrosome amplification (CA), in conjunction with mechanisms that ensure mitotic clustering of supernumerary centrosomes, is a key driver of chromosomal instability (CIN) in tumors. Centrosome clustering also allows the tumor to navigate the perils that accompany the presence of excess centrosomes. CIN is the essential vehicle that allows the tumor to reach its goal of generating enough intratumoral heterogeneity (ITH) to handle selection pressures and allow the development of aggressive tumor phenotypes. The propensity of proliferative cells in a tumor to undergo frequent error-

prone mitoses is termed Mitotic Propensity and constitutes the vital engine that powers the vehicle of CIN and propels it toward ITH.

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