Conduits of Intratumor Heterogeneity: Centrosome Amplification, Centrosome Clustering and Mitotic Frequency

Vaishali Pannu

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CONDUITS OF INTRATUMOR HETEROGENEITY: CENTROSOE
AMPLIFICATION, CENTROSOE CLUSTERING AND MITOTIC FREQUENCY

by

VAISHALI PANNU

Under the Direction of Ritu Aneja, PhD

ABSTRACT
Tumor initiation and progression is dependent on the acquisition and accumulation of multiple
driver mutations that activate and fuel oncogenic pathways and deactivate tumor suppressor
networks. This complex continuum of non-stochastic genetic changes in accompaniment with
error-prone mitoses largely explains why tumors are a mosaic of different cells. Contrary to the
long-held notion that tumors are dominated by genetically-identical cells, tumors often contain
many different subsets of cells that are remarkably diverse and distinct. The extent of this
intratumor heterogeneity has bewildered cancer biologists’ and clinicians alike, as this partly
illuminates why most cancer treatments fail. Unsurprisingly, there is no “wonder” drug yet
available, which can target all the different sub-populations including rare clones, and conquer the war on cancer. Breast tumors harbor ginormous extent of intratumoral heterogeneity, both within primary and metastatic lesions. This revelation essentially calls into question mega clinical endeavors such as the Human Genome Project that have sequenced a single biopsy from a large tumor mass thus precluding realization of the fact that a single tumor mass comprises of cells that present a variety of flavors in genotypic compositions. It is also becoming recognized that intratumor clonal heterogeneity underlies therapeutic resistance. Thus to comprehend the clinical behavior and therapeutic management of tumors, it is imperative to recognize and understand how intratumor heterogeneity arises.

To this end, my research proposes to study two main features/cellular traits of tumors that can be quantitatively evaluated as “surrogates” to represent tumor heterogeneity at various stages of the disease: (a) centrosome amplification and clustering, and (b) mitotic frequency. This study aims at interrogating how a collaborative interplay of these “vehicles” support the tumor’s evolutionary agenda, and how we can glean prognostic and predictive information from an accurate determination of these cellular traits.

INDEX WORDS: Mitotic frequency, Intratumoral heterogeneity, Metastasis, Centrosome amplification
CONDUITS OF INTRATUMOR HETEROGENEITY: CENTROsome
AMPLIFICATION, CENTROsome CLUSTERING AND MITOTIC FREQUENCY

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December 2014
DEDICATION

This thesis in its entirety is dedicated to my parents who have been an immense source of persistent love, direction and support. Without their extreme cooperation, patience, calmness and critical thinking, I could not even endeavor this task.

My parents – the two most significant people in my life, not only gave me life but also ensured shaping my future by inculcating strong values in me, which I stand by to this day. They taught me to work hard, be determined towards my goals and give more than hundred percent to the tasks I love to do. Their unfailing support and encouragement in the most demanding circumstances and constant emphasis on good education were the main drivers all these years. I would like to express my immense gratitude and love to my dearest brother, for his constant understanding and support. He has always been an invisible wall that I could lean on whenever I needed to and inspired me to bounce back with greater energy.

Last but not the least I thank each and every member of my family since a great family is that strong foundation on which one’s whole life rests.
ACKNOWLEDGEMENTS

As I stand at the threshold of earning my doctorate degree, I recall the numerous people who have helped me get this far. The list is long. I would like to firstly thank my advisor Dr. Ritu Aneja for her incessant support, direction and guidance throughout the course of my research. She has indeed been a great source of inspiration making me strive for better each time. I would also like to thank the members of my committee Drs. Jaideep Chaudhary, Zhi-ren Liu and Adam Marcus for their advice, support and time. Last but not the least; I thank all members of Dr. Aneja’s lab especially Padmashree CG Rida, Sushma Reddy Gundala, Angela Ogden, Sergey Klimov, Karuna Mittal and Alice Cheng, for their support.

This dissertation is not just a compilation of all my research work in Dr. Ritu Aneja’s lab, but is the fruit of innumerable blessings and good wishes from my closest family and friends.
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1 INTRODUCTION

Background and Significance

Breast tumors display startling intratumoral heterogeneity (ITH), both within primary and metastatic lesions. This revelation essentially calls into question mega clinical endeavors such as the Human Genome Project that have sequenced a single biopsy from a large tumor mass disregarding the fact that a single tumor mass comprises of cells that present a variety of flavors in genotypic compositions. It is also becoming recognized that this intratumor diversity underlies therapeutic resistance. Thus to comprehend the clinical behavior and therapeutic management of tumors, it is imperative to recognize and understand how intratumor heterogeneity arises. While personalized medicine involving the use of targeted therapy has revolutionized cancer medicine, its full potential is unlikely to be realized until the concept of intratumor genetic heterogeneity is factored in. The effectiveness of rationally designed tailored treatment hinges on our ability to comprehensively profile intratumor genetic and phenotypic heterogeneity.

To this end, my research proposes to study two main features/cellular traits of tumors that can be quantitatively evaluated as “surrogates” to represent intratumor heterogeneity at various stages of the disease: (a) centrosome amplification and clustering, and (b) mitotic frequency. We aim at interrogating how a collaborative interplay of these “vehicles” support the tumor’s evolutionary agenda, and how prognostic and predictive information can be gleaned from an accurate determination of these cellular traits. Outlined below is a brief synopsis of these cellular traits.
1.1 Centrosome amplification as a surrogate of Intratumor heterogeneity

Centrosome amplification, a long-standing cancer cell-specific trait is well known to compromise mitotic fidelity resulting in chromosomal instability that underlies the emergence of a variegated spectrum of genotypically and phenotypically distinct subtypes\textsuperscript{2-3}. Thus centrosome amplification serves the cancer cell by offering it a means to spawn an assortment of clones that propagates tumor evolution and forms the basis of intratumor heretogeneity. This idea is in complete consonance with the fact that centrosome amplification has long been correlated tightly with cancer aggressiveness. On this note, we rationally reason that centrosome amplification, a quantifiable cell-biological feature, may endow cells with cytoskeletonally-derived mechanical advantages to propel faster which might explain why certain non-invasive lesions transform into highly aggressive tumors with metastatic capabilities while others tend to remain indolent. A closer scrutiny of the phenomenon underlying this hypothesis has revealed that cancer cells harboring extra centrosomes passage through a transitory multipolar spindle state; after which they cluster their extra centrosomes to yield a pseudo-bipolar configuration, which sets the stage for chromosomal mis-segregation. During the dynamic transformation of the transient multipolar state into one with centrosomes “huddled” at the two poles, merotelic and syntelic kinetochore-microtubule (MT) attachments occur, thus engendering low-grade whole chromosome missegregation. This incessant generation of low-grade aneuploid clones ensures that the cancer cells do not “rock the boat” too much to threaten their own survival. This fosters tumor evolution through clonal cooperation, a new paradigm that questions the widely prevalent notion of Darwinian tumor evolution, which supports this relentless process of clonal evolution until a clone with a superlative karyotypic composition is created which then takes over.
1.1.1 *Centrosome: Microtubule-organizing center of the cell*

A centrosome is a non-membranous organelle composed of a pair of orthogonally-arranged centrioles embedded in a cloud of pericentriolar material (PCM). The pair of centrioles, linked by a loose linker, are essentially a mother and daughter centrioles that differ in their extent of maturity.\(^4\) PCM is composed of over 200 centrosome-associated proteins including γ-tubulin ring complexes (γ-TuRCs) and the MTs they nucleate, microtubule-associated proteins (MAPs), Homologous to AUgmin Subunits (HAUS) complexes, and several other integral and satellite proteins, collectively providing the site for MT nucleation and thus, determines the MT density in the cell.\(^5\) The mother centriole regulates PCM organization, stabilization and size.\(^6-7\) The centrosome, thus congregates the cytoskeletal network by MT nucleation, anchoring, and radiating MT fibers when required.\(^8\)

Typically, the centrosome duplicates precisely once before the onset of mitosis, in S-phase, concomitant with DNA replication.\(^9\) During mitosis, the duplicated pair of centrosomes anchor a radial array of astral, kinetochore, and interpolar MTs, constructing a fusiform spindle apparatus with the help of various MAPs and motor proteins.\(^10\) As any defects in mitotic spindle organization may lead to catastrophic consequences, centrosome structure, duplication and function are very tightly regulated inside a cell.

1.1.2 *How cells generate extra centrosomes*

Centrosome amplification, referring to an increment in the centrosome number per cell, can ensue as a result of diverse cellular events, such as cell fusion, PCM or centriole fragmentation, *de novo* centriole formation, dysregulated centrosome duplication cycle, and possibly cytokinesis failure.\(^11-13\) Besides numerical aberrations, centrosome amplification frequently entails structural as well as functional abnormalities in centrosomes.\(^14-16\) One of the
most emphasized mechanisms of centrosome amplification is deregulation of the centrosome duplication cycle owing to the fact that various oncogenic proteins are associated with regulating centrosome duplication. An alternative crucial means of centrosome amplification depends upon compromising ‘licensing' mechanisms that couples the centrosome duplication cycle with the cell cycle. ‘Licensing’ system ensures that centriole duplication is only initiated when appropriate, that is, when only one pair of centrioles is present, and the cell is ready to divide. Substantial body of evidences attests the dysregulation of centrosome licensing factors in a wide range of cancers. For instance, separase overexpression occurs in several cancers and correlates positively with tumor grade and negatively with survival. Plk1 upregulation is so widespread in malignancies that it has been termed a ‘general characteristic of human cancer.'

Centriole overduplication is a significant but, by no means the only possible cause of centrosome amplification. Convincing line of evidence reveal that DNA damage caused due to factors like ionizing radiation or aberrant DNA repair mechanisms, are entirely capable of inducing centrosome amplification in cancer cells by means of a sustained S/G2 cell-cycle arrest.

1.1.3 The problem with centrosome amplification

The presence of excessive number of centrosomes within a cell sets the stage for rampant spindle multipolarity during metaphase, thus increasing the errors in spindle poles attachments which sister chromatids. The resultant multipolar cell division leads to the generation of more than two daughter cells that may be inviable owing to catastrophic levels of chromosomal loss (viz, death-inducing, high-grade aneuploidy).
1.1.4 Managing extra centrosomes by centrosome clustering

Despite the catastrophic consequences of centrosome amplification, cancer cells frequently harbor supernumerary centrosomes and yet manage to thrive. It is becoming increasingly evident that cancer cells hijack cell-cycle specific mechanisms and strategically employ them to manage extra centrosomes by clustering them into two spindle poles.\textsuperscript{22-24} This clustering of numerous centrosomes roughly at two poles leads to the formation of a ‘pseudo-bipolar’ mitotic spindle, which circumvents multipolarity. A detailed dissection of the clustering phenomenon reveals the existence of a transient multipolar spindle intermediate prior to the centrosome clustering mechanism ensues.\textsuperscript{25-26} During this transient multipolar state, merotelic kinetochore–MT attachments (i.e., attachment of MTs from two different spindle poles to the same kinetochore) take place, which are poorly sensed by the spindle assembly checkpoint (SAC). Such attachments are known to severely compromise genomic integrity and promote CIN by causing both structural and numerical karyotypic abnormalities.\textsuperscript{27} The consequent low-grade aneuploidy is not merely an undesirable-but-benign consequence of cellular transformation but rather a characteristic that actually drives malignancy and tumor evolution.\textsuperscript{28} This finding is corroborated by voluminous evidence that centrosome amplification is an early event in carcinogenesis, as it occurs in precancerous and preinvasive lesions.\textsuperscript{15, 29}

1.1.5 Players involved in centrosome clustering

1.1.5.1 SAC

The SAC normally prevents anaphase entry until all chromosomes have bioriented on the mitotic spindle.\textsuperscript{30-32} The SAC-mediated delay in anaphase onset also provides time for effecting centrosome clustering, thereby promoting pseudo-bipolar mitosis and daughter cell survival. The following observations support the idea that the SAC facilitates centrosome clustering. SAC-
mediated, delayed passage through mitosis allows Plk4-overexpressing fruitfly larvae, which exhibit centrosome amplification, to cluster their extra centrosomes. Moreover, in near tetraploid Drosophila S2 cells, which naturally exhibit centrosome amplification, clustering is inhibited by disabling SAC function through Mad2 knockdown. However, when Mad2 knockdowns are treated with the proteasome inhibitor MG132, which delays anaphase onset, clustering is accomplished. These data bolster the notion that the SAC is required for clustering because it delays anaphase onset, which provides extra time for centrosome clustering.

1.1.5.2 Actin cytoskeleton

One mechanism, whereby actin promotes clustering, is by mediating cortex-oriented centrosome migration during mitosis – a process that is dramatically impaired upon actin depolymerization. Cortical ‘cues’ established during interphase direct the positioning of the mitotic spindle and centrosomes during mitosis. Despite the anonymity of most of these cues, it is evident that interphase cell-adhesion pattern contributes to their establishment within the cortical actin meshwork. For instance, plating breast cancer MDA-MB-231 cells with amplified centrosomes on a fibronectin micropattern with a single long axis results in spindle orientation along this axis, whereas plating on an ‘O’ shaped micropattern results in random spindle orientation. Cells plated on a micropattern with a single long axis, but treated with an inhibitor of cortical asymmetry, are less able to align their spindle along this axis or to cluster centrosomes. Altogether, these experiments suggest a crucial role of interphase cell-adhesion pattern in the asymmetric distribution of cortical cues (e.g., at opposite cellular poles) to promote centrosome positioning, clustering, and consequent spindle bipolarity.
1.1.5.3 Microtubule motors

1.1.5.3.1 HSET

The Kinesin-14 family member, HSET (also known as KIFC1), a minus end-directed microtubule motor, has a critical role in centrosome clustering in some cell lines. Human HSET localizes between microtubules within the mitotic spindle. HSET inhibition has no significant impact on bipolar anaphase or cell viability in human BJ fibroblasts, which exhibit virtually no centrosome amplification (~1%), as well as mouse NIH-3T3 fibroblast and human MCF-7 breast cancer cells, which exhibit only ‘low-level' centrosome amplification. However, in cells with a higher incidence of centrosome amplification, such as isogenic tetraploids of the aforementioned cell lines or MDA-MB-231 cells, bipolar anaphase is inhibited and viability compromised by HSET knockdown. These data suggest that the presence of a ‘normonumerary' centrosome complement partly masks the requirement for HSET in spindle organization. Although HSET appears to serve no vital function when centrosome number is normal, the minus-end-directed motor was originally identified in embryonic mouse brain; consequently, the possibility that the protein may serve a non-redundant function during embryogenesis cannot be excluded. Although HSET knockdown in HeLa cells does result in a minor attenuation of spindle pole focusing, central spindle stability, and microtubule density, along with a mild augmentation of spindle breadth, the occurrence of a bipolar anaphase remains unimpeded. HSET inhibition also results in shortening of the spindle in HeLa cells, suggesting that HSET promotes spindle lengthening and pole separation.

In normal cells, the two centrosomes mask the requirement for HSET in normal spindle biogenesis. In certain transformed cell lines, HSET and NuMA play overlapping and therefore redundant cellular functions, in which case HSET may be non-essential for centrosome
Differential dependence on HSET may indicate that various cell types have evolved distinct clustering mechanisms. Owing to the seemingly nonessential role of HSET in nontransformed human cells, HSET offers immense promise as a novel chemotherapeutic target for ‘centrosome-rich’ cancers, including those of the breast, prostate, bladder, colon, and brain.

1.1.5.3.2 Dynein

The role of dynein in centrosome clustering appears to be complex and somewhat elusive. In some cancerous (UPCI:SCC114 oral squamous cells) and noncancerous (Colcemid-treated or hMps1-overexpressing HEK293) cells with supernumerary centrosomes, displacement of dynein from the spindle results in centrosome declustering. Thus, it appears that spindle-localized dynein is required for centrosome clustering in these cell lines.

The prediction that dynein may have a role in centrosome clustering would be commensurate with dynein's extensive involvement in mitotic progression, centrosome assembly and localization, spindle organization, and positioning. Dynein at the kinetochore may be involved in kinetochore–microtubule attachment and SAC silencing. The dynein/dynactin complex not only transports a variety of proteins (including several others that promote microtubule nucleation) to the centrosome but also nucleates microtubules itself. Dynein/dynactin is also necessary for centrosomal retention and focusing of microtubules, regulation of mitotic spindle positioning and length, and migration of interphase cells.

1.1.5.4 MT dynamicity

Extensive research in recent years has turned the spotlight onto a property of microtubules that is central to their biological function, viz, their dynamic instability, as a key requirement for effective clustering of supernumerary centrosomes. Treatment of cells exhibiting
amplified centrosomes with drugs that gently attenuate microtubule dynamicity, such as griseofulvin\textsuperscript{51} or bromonoscapine,\textsuperscript{52} induces declustering of supernumerary centrosomes, indicating that microtubule dynamicity is an important requisite for centrosome clustering. Although centrosome declustering drugs are very different in many respects – for instance, griseofulvin is an antifungal whereas bromonoscapine is a derivative of the antitussive drug, noscapine – the attribute they share is the ability to subtly attenuate microtubule dynamic instability and selectively kill cancer cells. There is ample empirical evidence to support the idea that tension at the kinetochore is a crucial clustering mechanism. There has recently been identified a host of proteins necessary for clustering in oral squamous cancer UPCI:SCC14 cells.\textsuperscript{23} Among these were many proteins involved in generating and/or sensing tension at the kinetochore. For instance, clustering was inhibited by knockdown of certain Ndc80 proteins (viz, HEC1, SPC24, and SPC25) and shugoshin. The Ndc80 complex, part of the outer kinetochore, is involved in not only capturing kinetochore microtubule plus-ends but also coupling the force of their depolymerization to chromosomal movement.\textsuperscript{53}

1.1.6 Targeting centrosome clustering for chemotherapy

Too many centrosomes may prove bane or boon to cancer cells depending on whether the cell is able to cluster them neatly at opposite poles. Clustering may confer survival advantages and promote malignancy by predisposing the cell to CIN via merotelic microtubule–kinetochore attachment and genome missegregation.\textsuperscript{25-26} When ‘low-grade’ (i.e., survivable) missegregation results in the loss of a gene that promotes faithful chromosome segregation and maintenance (or gain of another copy of a gene that disturbs these processes), then the cell acquires CIN – essentially, the ability to shuffle its genome until a stable, malignant phenotype is procured.\textsuperscript{54} By contrast, in the absence of clustering, supernumerary centrosomes result in spindle multipolarity,
which may cause aneuploidy of a mortally high grade. Alternatively, multipolar cells may arrest in mitosis and succumb to death via other mechanisms.\textsuperscript{26,52} Given the lethality of the multipolar state, induction of high-grade spindle multipolarity constitutes a novel chemotherapeutic strategy whose efficacy holds much promise. Moreover, declustering of supernumerary centrosomes to achieve multipolarity should specifically target cancer cells and pose no apparent threat to most healthy tissues, unlike the majority of current anticancer remedies, drugs and radiation alike. Understanding the mechanisms by which the few known declustering agents operate can pave the way for rational design and synthesis of cancer cell-specific and thus ‘kinder and gentler’ chemotherapy.

1.2 Mitotic frequency as a surrogate of Intratumor heterogeneity

Chromosomal instability (CIN) in cancer cells is associated with changes in chromosome structure and number that result in increased segregation errors during cell division and promote ITH in solid tumors. Since CIN (and the consequent aneuploidy) is caused by aberrant chromosome segregation, it is probable that most cancer cells acquire defects in the machinery responsible for faithful chromosome segregation in mitosis. These defects could arise through either mutation of genes encoding essential mitotic proteins or by imbalances in protein levels or activities that reduce mitotic fidelity.\textsuperscript{55-56}

CIN is a high rate of chromosome mis-segregation that enhances karyotypic diversity in cells within the same tumor, a feature commonly associated to tumor aggressiveness.\textsuperscript{57-58} On average, CIN cancer cells mis-segregate a chromosome once in every one to five cell divisions,\textsuperscript{2,} an event which is thought to drive the genomic re-shuffling and ITH that allows cells to acquire new phenotypes such as drug resistance.
1.2.1 Defects in spindle assembly checkpoint

SAC ensures the accurate partitioning of genomic material during cell division by inhibiting anaphase onset until all the chromosomes in the dividing cell are properly attached to the spindle MTs. As even a single mis-segregation event can result in altered chromosome numbers or aneuploidy (a condition frequently observed in cancer cells), checkpoint regulation is a crucial factor dictating the genetic fidelity of cell division. Rather than responding to a ‘proceed to anaphase’ signal and somehow measuring a signal generated when all 46 chromosome–microtubule attachments are established, the SAC responds to a ‘stop anaphase’ signal that is generated by unattached kinetochores.

The main elements of the SAC signaling network concertedly form a mitotic checkpoint complex (MCC) that facilitates inhibition of the anaphase-promoting complex/cyclosome (APC/C)-dependent ubiquitylation and subsequent proteolysis of substrates, including securing and cyclin B (cell-cycle-progression regulatory proteins). Each unattached kinetochore catalyzes a conformational change in the spindle checkpoint protein Mad2 so as to bind cell-division cycle protein 20 (Cdc20), and, together with the mitotic checkpoint proteins Bub3 and BubR1, forms the MCC.

Normal cells have a robust mitotic checkpoint in which one or more unattached kinetochores can produce a signal that is strong enough to inhibit all cellular APC/C activity and thereby block progression to anaphase. This is not necessarily true when checkpoint components are mutated or their concentrations are reduced. While complete loss of the SAC activity is lethal due to massive mis-segregation, partial loss of the SAC is a common feature of many aneuploid tumor cells allowing them to gain or lose a small number of chromosomes. Several studies have
reported that human tumor cells contain mutations in mitotic checkpoint genes that encode BUB1, BUBR1, MAD1, MAD2.\textsuperscript{36-38, 61-62}

The involvement of SAC in generating genomic instability stemmed from a study demonstrating that CIN is caused by mutational inactivation of the essential SAC component Bub1.\textsuperscript{63} Although the initial identification of mutations in Bub1 was encouraging, subsequent genome sequencing showed that mutational inactivation of SAC components is quite rare.\textsuperscript{64} Moreover, complete loss of SAC function is lethal.\textsuperscript{65-66} Also, follow up experiments demonstrated that most aneuploid cancer cells possess a functional SAC.\textsuperscript{67} These results gave rise to the alternative hypothesis that partial loss of SAC function is responsible for causing CIN. Evidence in favor of this view is derived from the high incidence of aneuploidy and tumorigenesis in mice engineered to have weakened SAC activity.\textsuperscript{68}

The evidences provided by several studies, taken together, indicate that there are several ways to slip past the spindle assembly checkpoint. First, it has been shown that merotelic attachments (when one kinetochore is attached to both spindle poles) do not generate a checkpoint response and can lead to ‘lagging’ chromosomes that get left behind during anaphase, typically near the middle of the dividing cell. Second, if a chromosome detaches from the spindle too close to anaphase, there is insufficient time to block the cell cycle and prevent mis-segregation. Third, a cell that cannot properly regulate the total and relative levels of the SAC proteins may fail to ensure error-free chromosome segregation.\textsuperscript{69}

So, whereas an initial weakening of the mitotic checkpoint might increase the chances of CIN-mediated tumorigenesis, further weakening (or silencing) of checkpoint signaling results in cell autonomous lethality. This raises the prospect of manipulating the mitotic checkpoint to
inhibit tumor cell proliferation with drugs that target essential checkpoint functions, such as BUBR1 kinase activity, to treat certain cancers.\textsuperscript{66}

1.2.2 \textit{Chromosome segregation errors}

Faithful chromosome segregation relies on the bi-oriented attachment of chromosomes to spindle microtubules. Chromosome segregation errors during anaphase fall into three subtypes that arise from distinct mechanisms. Errors in mitotic spindle function generate lagging chromosomes\textsuperscript{2}, whereby an entire chromosome fails to segregate properly by virtue of its attachment to microtubules emanating from opposite spindle poles. Lagging chromosomes are a hallmark of whole chromosomal instability (w-CIN).\textsuperscript{70} Anaphase spindles can also exhibit bridged chromatin (Murnane, Mutat Res, 2012) such that DNA, from the same chromosome or from non-disjoined sister chromatids, is stretched towards opposite spindle poles. The third class of segregation errors consists of acentric chromatin fragments that are devoid of centromeres and thus cannot establish canonical kinetochore-microtubule (k-MT) attachments to the mitotic spindle. Chromatin bridges and acentric chromatin fragments are hallmarks of structural chromosomal instability (s-CIN). Merotely is a specific k-MT attachment error defined by single kinetochores that simultaneously attach microtubules oriented toward both spindle poles. Merotely avoids detection by the SAC since kinetochores attain full occupancy of microtubules (albeit with improper orientation). Thus, if cells enter anaphase with merotelically attached kinetochores, the chromatid attached to both spindle poles will fail to segregate properly and lag near the central spindle as other chromosomes move poleward. This can cause chromosome mis-segregation and lagging chromosomes in anaphase were the most common defect in mitosis observed by live cell imaging in cancer cell lines.
1.2.3 Tumor Proliferation as a Prognostic Marker

Tumor proliferation is a critical clinical parameter that pathologists essentially consider for tumor grade determination. Proliferation rate is currently measured by assessing mitotic cell number or percentage Ki67 positive tumor nuclei.

Mitotic index (MI), determined by counting number of mitotic cells per 10 HPFs in the tumor tissue, has been shown to carry a strong prognostic value. MI has a limited reflection on the doubling time of the tumor. However, mitotic frequency bears strong implications on tumor progression and aggressiveness. Literature reports indicate that some early stage tumors may divide rapidly to allow chromosomal instability to enable genetic diversity facilitating selection of superlative karyotypes. Thus, the higher the mitotic frequency within a tumor, the higher the probability of emerging aggressive clones that fuel tumor progression. Mitotic score within an early-stage tumor is therefore a crucial contributor to the risk of acquisition of aggressive phenotypes.

Nuclear Ki67 positivity is defined as the percentage of stained carcinoma cells. Ki67 protein is present during all cell cycle phases (G1, S, G2 and M) and is strictly a cell proliferation marker. Ki67 is highly recommended for distinguishing between “luminal A” and “luminal B” subtypes in breast cancer patients. However, clinical arena still lacks consensus on standard Ki67 assessment protocols and cut-off values, thus making it a rather poorly reproducible index of tumor proliferation. Various studies have established statistically significant association between Ki67 and breast cancer prognosis except that the cut-offs used in the studies to categorize “high” and “low” Ki67 groups varied from 1% to 28.6%, indicating the unreliability of its clinical significance.
A mitotic frequency-based risk index strictly relies on the relative estimation of mitotic and Ki67 positive cells within a tissue sample. Thus, it requires accurate assessment of both the indices in the same field, on the same scale. A major impediment to developing this index is the fact that Ki is customarily assessed as a percentage out of a preset number of nuclei counted and MI is assessed as number of mitotic figures present in a preset number of microscopic fields. In addition, Ki67 positivity is quantitated in a low-power microscopic field whereas mitotic cells are identified in a high-power microscopic field. Thus, current clinical methods lack the accuracy and depth of analysis required for precise estimation of mitotic frequency within a proliferating tumor.

1.3 References


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2. RAMPANT CENTROSOME AMPLIFICATION UNDERLIES A MORE AGGRESSIVE DISEASE COURSE IN BREAST CANCERS

2.1 Abstract

Centrosome amplification (CA) is a long-standing cancer cell-specific trait. CA occurs in pre-invasive lesions implicating an early and causal role of excess centrosomes in driving tumor progression. Recent studies provide convincing evidence that CA leads to malignant transformation and promotes invasion in mammary epithelial cells. Given that supernumerary centrosomes occur in ~80% of breast cancers, we hypothesize that CA can serve as a predictor of breast cancer aggressiveness. Triple (ER/PR/Her2) negative (TN) breast cancer is an aggressive histological breast cancer subtype that commonly afflicts African American (AA) women and is characterized by high recurrence, metastases, and mortality rates. TN and non-TN breast tumors follow variable kinetics of metastatic progression, and thus present an ideal model system to explore if the severity and nature of CA can distinguish the two subtypes. Utilizing formalin-fixed paraffin-embedded and fresh-frozen breast cancer tissues, we quantitatively assessed the extent of structural and numerical centrosomal aberrations for each patient sample in a large cohort of grade-matched TNBC and non-TNBC cases, using multi-color immunofluorescence confocal imaging and immunoblotting. Our data establish previously unrecognized differences in incidence and severity of CA between TNBC and non-TNBC cell lines as well as in grade-matched patient samples. We also found strong correlation between CA and markers of aggressiveness associated with breast cancer metastasis. Using time-lapse imaging in MDA-MB 231 cells stably transfected with GFP-tagged centrin harboring amplified centrosomes, we demonstrate that robust CA is associated with enhanced migratory ability in cells. Collectively,
our data suggest that centrosome amplification status dictates migratory potential in cells and the extent of this organellar abnormality perhaps drives aggressive phenotypes in TNBCs. This organellar disparity at the centrosome level has immense translational potential as it may allow early risk prediction and explain higher tumor aggressiveness, and mortality rates in TNBC patients. Our study thus fulfills a vital knowledge gap by identifying cell biological characteristics underlying breast cancer aggressiveness.

2.2 Introduction

Triple-negative breast cancers (TNBC), negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), are characterized with aggressive behavior, poor prognosis with high recurrence rate and lack of targeted therapies. On account of these attributes, TNBCs are currently at the prime target of clinical and biological research.

Although various studies suggest that TNBCs primarily are chemo-sensitive and tend to show elevated pathological complete response (pCR) to neoadjuvant chemotherapy, they are characterized with poor survival owing to higher disease relapse following chemotherapy.

In breast cancer diagnosis, hormone status (ER/PR) presented as one of the earliest prognostic markers that led to the development of hormone-based therapy for breast cancer. The subsequent establishment of HER2 overexpression as a marker for targeted breast cancer treatment improved the outcomes associated with various breast cancer subtypes. However, current predictors of TNBC prognosis and treatment response are largely ill-defined, rendering the management of this unruly disease a considerable challenge.
Alternatively, recent studies have revealed that centrosome abnormalities and chromosome instability strongly characterize pre-invasive in situ ductal carcinomas, demonstrating the significance of these anomalies as early events in breast carcinogenesis.

Centrosome amplification (CA) has been significantly correlated with aneuploidy and chromosomal instability in invasive breast carcinomas. These evidences provide substantial grounds to investigate the role of centrosome amplification in predicting malignant potential of breast cancer cells.

BRCA1 and BRCA2 gene mutations are shown to have been associated with ~15% of TNBC, and in turn, TNBC accounts for 70% of breast tumors carrying BRCA1 mutation and ~20% of them with BRCA2 mutation. It is well established that BRCA1 and BRCA2 tumor suppressor genes directly dictate chromosome stability by regulating DNA repair and p53-mediated cell cycle checkpoint control as well as centrosome duplication cycle. These findings establish the causative link between BRCA1 and BRCA2 mutations and extensive CIN found in TNBC patients. Furthermore, TNBC tumors frequently overexpress cyclin E, resulting in aberrant activation of cyclin E/Cdk2 complex, one of the key players dictating the induction of centrosome amplification, eventual genetic instability and ultimately culminating into enhanced breast cancer progression. Therefore, CA may present a fascinating prognostic and therapeutic target for the management and treatment of TNBCs, currently suffering from insufficient therapeutic molecular targets.

Thus far, a thorough quantitative comparison of centrosomal aberrations in breast tumors subtypes with inherently different metastatic capability has never been reported. In this study, we performed comprehensive quantitative analysis of centrosomal abnormalities in breast tumors to establish differences in incidence and severity of CA (structural and numeral) between grade-
matched TNBC and non-TNBC patients and were able to significantly correlate CA status with patient outcomes. We also established link between CA markers and markers of breast tumor aggressiveness and consequently demonstrating that robust CA underlies acquisition of migratory and invasive abilities in vitro.

Through this study, we aim to establish CA as a quantifiable property of early-stage tumors that can predict the risk of a tumor being or becoming an aggressive one. Providing clinicians with a method to stratify early-stage tumors into high- and low-risk subgroups would enable channeling of patients into optimal treatment paths and abridge existing disparities in breast cancer patient outcomes.

2.3 Materials and Methods

2.3.1 In silico analysis:

I. Data Collection:

One channel micro array data were collected from Gene Expression Omnibus (GEO) database and Cancer Genome Atlas (TCGA). The list of the GSE ID's are given in supplementary table.

II. Data pre-processing:

One channel micro array data was Mas5.0 normalized [3], and was further taken for processing.

III. Centrosomal Amplification Index (CAI) Calculation:

Logarithm to the base 2 transformed expression levels of PLK4, Aurora-A, Centrin, \(\gamma\)-tubulin and pericentrin genes from breast cancer patients were extracted from the TCGA and
GEO patients and a summation was calculated to generate a CAI for TNBC and Non-TNBC patients.

IV. CAI and associated clinical outcome information:

Associated grade, stage and clinical information for patients from GEO and TCGA database were plotted for CAI expression levels.

2.3.2 Clinical tissue samples:

For this study paraffin embedded slides were procured from Gabriela Oprea, Grady Memorial Hospital and Northside Hospital with information on clinical outcomes. The Emory Institutional Review Board (IRB) and Northside Hospital approval was obtained for all aspects of the study.

2.3.3 Immunofluorescence staining, imaging and scoring:

For Immunofluorescence staining all tissue slides were deparaffinized by baking at 67 degree Celsius for 2 hours followed by 3 xylene washes. Slides were then rehydrated by passing them through a series of ethanol baths (100%, 95%, 70% and 50%). Antigen retrieval was performed by citrate buffer (pH 6.0) in a pressure cooker (15 psi) for 3min. Tissue samples were than incubated for overnight with primary antibody against gamma tubulin dilution was 1:1000 in 4 degree. The samples were washed with 3x PBS before incubating them for secondary (alexa-488 anti-mouse) at 37 degree for 2 hours. Samples were washed with 3x PBS and then mounted with Prolong-Gold antifade reagent that contained DAPI (Invitrogen). Tissue samples were imaged using the Zeiss LSC 700 microscope Confocal microscope (Oberkochen, Germany) and images were processed with Zen software (Oberkochen, Germany). Percentage cells with centrosomal abnormality were quantitated from 10 randomly selected fields per sample (500 cells were counted for each sample)
2.3.4 **Immunohistochemistry, scoring and statistics:**

Samples were processed in the same way till antigen retrieval as mentioned above in Immunofluorescence. Tissues were then immunostained for Vimentin, Plk4 and Aurora- A. To perform enzymatic antibody detection Universal LSAB + kit/HRP (DAKO, CA, USA) was used. Vimentin staining was quantified for both the nuclear and cytoplasmic localization as an intensity and frequency score. A relative intensity score was represented as 0 = none, 1 = low, 2 = moderate, or 3 = high and frequency score was depicted as the percentage of cell nuclei or cytoplasm demonstrating Vimentin positivity (i.e. a score of 1, 2, or 3). The product of the relative intensity and frequency was measured as Vimentin weighted index (WI) for both the nucleus and cytoplasm. Vimentin WI was then compared for TNBC and non- TNBC tissue samples.

2.3.5 **Immunoblotting and antibodies:**

Protein lysates were prepared from ~70% confluence cells and frozen tissue samples (sonicated). Immunoblotting was performed as described earlier\(^1\). Briefly, PAGE was used to resolve the proteins and transferred on to PVDF membrane (Millipore). Pierce ECL chemiluminescence detection kit (Thermo Scientific) was used to visualize the immune-reactive bands corresponding to respective primary antibodies. β-actin was used as loading control. For immunofluorescence staining, cells were grown on glass coverslips and fixed with ice cold methanol for 10 min. Blocking was done by incubating with 2% bovine serum albumin/PBS.0.05% Triton X-100 at 37 °C for 1h. Primary Antibodies against γ-tubulin, α-tubulin were incubated with coverslips for 1h at 37 °C at the dilution 1:2000.The cells were washed with 2% bovine serum albumin/PBS for 10 min at room temperature before incubating
with a 1:2000 dilution of Alexa 488- or 555-conjugated secondary antibodies Invitrogen (Carlsbad, CA, USA). Cells were mounted with Prolong Gold antifade reagent that contains 4′,6-diamidino-2-phenylindole (Invitrogen). Antibodies against γ-tubulin, α-tubulin and Vimentin were from Sigma (St. Louis, MO, USA), Aurora-A, Plk4 and β-actin were from cell signaling, Centrin-2 and Cyclin-E from Santacruz and Pericentrin-2 from Abcam. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4 Results

2.4.1 Overexpression of CA genes is associated with reduced survival and TNBC status in silico

In solid tumors, centrosomal abnormalities are associated with advancing stages of the disease, aneuploidy, and an aggressive clinical course. Given that there is a wide divergence in aggressiveness between TNBC and non-TNBC patients, we reasoned that expression levels of centrosomal genes might offer valuable insights into the disparate behavior between the two histological subtypes. To this end, we mined publically-available microarray data of breast cancer patients to evaluate gene expression levels for major structural centrosomal proteins, both centriolar (centrin) and pericentriolar (pericentrin and γ-tubulin). In an attempt to gain a more complete picture of centrosomal aberrations, we included genes implicated in centrosome amplification (Polo-like kinase 4 and Cyclin E). We calculated a gene expression-based centrosome amplification index (CAI) by adding log transformed, normalized gene expression for CETN2 (centrin-2), TUBG1 (γ-tubulin), PCNT2 (pericentrin), PLK4 (polo-like kinase 4) and CCNE1 (cyclin E) genes. A panel of five centrosomal genes was selected instead of a single gene because cancer is a clonally evolving disease and centrosome amplification could arise due
to dysregulation of different genes in different cancers and even cancer cell clones. First, we evaluated the relationship of higher centrosome amplification, as assessed by CAI, with disease aggressiveness, as evaluated by overall survival (OS). OS was calculated as the number of days from diagnosis to death or last follow-up if death was not recorded. Irrespective of receptor status (TNBC n=101, non-TNBC n=61), patients with higher CAI had lower OS (p=0.048) than patients with lower CAI (Fig.2.4.1A).

Further analysis of a dataset of 138 TNBC and 466 non-TNBC samples clearly showed significantly higher CAI in TNBCs compared to non-TNBCs, even when they were (a) grade matched (Fig.2.4.1B), or (b) stage matched (Fig. 2.4.1C).

Figure 2.4.1 Breast tumors with high CAI have lower survival rate compared to low CAI tumors.
A. Overall survival plots for high and low CAI groups. CAI (CA genes) is higher in in silico TNBC (n=84) compared to in silico non-TNBCs (78). B. Box whisker plot comparing the CAI (CA genes expression) in TNBC and non-TNBC patients considering grades (p<.001 for grade matched TNBC and non-TNBC patients). C. Box whisker plot comparing the CAI (CA genes expression) in TNBC and non-TNBC patients considering stages (p<.005 for stage matched TNBC and non-TNBC patients).

2.4.2 TNBC patients have higher centrosome amplification than non-TNBC patients

Having found significantly elevated CAI in TNBC compared to non-TNBC, we microscopically visualized amplified centrosomes in grade-matched tissues from TNBC (n=59)
and non-TNBC (n=116) patients (Fig. 2). Centrosomes were labelled by \(\gamma\)-tubulin (green) staining, wherein centrosomal aberrations were determined by abnormal number of \(\gamma\)-tubulin spots (more than two) as well as by increased volume over normal centrosomal volume in breast epithelial cells. **Fig. 2.4.2A** shows representative confocal immunomicrographs show centrosomes (\(\gamma\)-tubulin, green) and nuclei (DAPI, blue) from normal breast tissue and grade-matched breast cancer tissue from TNBC and non-TNBC patients. Percentage of cells with more than two centrosomes (2 was quantitated by examining and counting centrosomes in at least 500 cells/slide. **Fig. 2.4.2Bi** shows that the number of cells harboring extra centrosomes was significantly higher in TNBC samples (62%, n=30) compared to non-TNBC (30%, n=98) (p<0.05) in grade-matched cases. We next determined the centrosomal volumes in all cases using the 3D measurement module from the Zeiss imaging software. While centrosomal volume in normal breast epithelial cells was 0.22 \(\mu \text{m}^3\), the mean volume of \(\gamma\)-tubulin-stained spots analyzed in at least 500 cells from each patient sample was 4.05 \(\mu \text{m}^3\), which is ~15 times higher than the centrosomal volume in normal cells. **Fig 2.4.2Bii** shows that centrosome volume was significantly higher in TNBC samples (average=6.8 \(\mu \text{m}^3\)) compared to non-TNBC samples (average=4.2 \(\mu \text{m}^3\)) (p<0.05) in grade-matched cases.
Figure 2.4.2 Centrosome amplification status in TNBC and non-TNBC patients.
A. Representative confocal micrographs depicting the status of centrosome amplification in grade1, grade 2 and grade3 TNBC and non- TNBC tissues. Centrosomes stained for γ-tubulin (green) and DNA for DAPI (blue). B. Bar graph representation of percent cells showing supernumerary centrosomes (>2) in TNBC and non-TNBC tissue samples. C. Box whisker graph representation of average volume of centrosomes in normal, TNBC and non- TNBC tissue samples. 500 cells were counted in each case.
We also evaluated expression levels of centrosomal proteins in fresh-frozen tumor and uninvolved adjacent tissue in 20 pairs of grade-matched samples from TNBC and non-TNBC patients. Immunoblotting of cell lysates showed higher expression of two centrosomal proteins (centrin-2 and γ-tubulin) in TNBC than in non-TNBC patients. We also observed higher levels of centrosome amplification protein (Plk4) in TNBC samples (Fig. 2.4.3Ai).

In addition, breast cancer cell lines derived from TNBC patients (MDA-MB-468, MDA-MB-231) showed (a) higher incidence and severity of centrosome amplification (Fig. 2.4.3Bi,ii) (b) elevated expression of centrosomal (pericentrin, centrin-2, γ-tubulin) and centrosome amplification (Plk4 and cyclin E) markers, compared to non-TNBC-derived (MCF-7) lines (Fig. 2.4.3Aii). Intriguingly, the extent of amplification was considerably lower in cell-lines (5-30%) than what was found in the patient tissue samples (15-80%), irrespective of the receptor status.
Figure 2.4.3 TNBC patients show higher expression of centrosomal markers.
Ai. Immunoblots for 5 paired breast tumor (T) and normal adjacent (N) tissues from grade and receptor matched TNBC and non-TNBC patients showing expression levels of centrosomal markers. ii. Immunoblots of centrosomal markers in MCF-7 (non-TNBC), MDA-MB-460 and MDA-MB-231 (TNBC cell lines). Bi. Immunofluorescence micrographs showing MCF-10A cells (normal), MCF-7 cells (non-TNBC) and MDA-MB-468 and MDA-MB-231 cells (TNBC) in interphase and mitotic state, stained for γ- tubulin (green) and DAPI (blue). ii. Bar graph
quantitation of percent cells depicting multipolar mitosis and centrosomal amplification in MCF-7 (non-TNBC) and MDA-MB-468 and MDA-MB-231 (TNBC) cell lines.

2.4.3 Centrosome amplification status correlates strongly with metastatic disease and progression-free survival

We next evaluated protein expression levels of centrosome amplification molecules by immunohistochemical staining on paraffin-embedded tumor and uninvolved adjacent tissue in 20 pairs of grade-matched TNBC and non-TNBC samples. Immunohistochemical data strongly suggested overexpression of well-established centrosome amplification markers (Aurora A, Plk4 and cyclin E) in TN compared to non-TN samples (Fig. 2.4.4A). We also immunostained the same samples with breast cancer aggressiveness marker (vimentin). As expected, TNBC samples exhibited significantly higher expression of vimentin (Fig. 2.4.4B). Representative images of two sets of breast tumor samples exhibiting varying expression levels of centrosomal proteins shown in blot in Fig. 2.4.4C, show a strong correlative pattern of Aurora-A and Plk4 with vimentin in both tumor and normal breast tissue (Fig. 2.4.4A). Statistical analysis of 20 pairs of grade-matched TNBC and non-TNBC samples revealed a strong positive correlation between Plk4 and vimentin (r=0.58, p<0.02). Furthermore, we evaluated the relationship of higher centrosome aberrations [as assessed by %CA in Fig. 2Bi with PFS in breast cancer patients. PFS was calculated as the number of days from diagnosis to the first local recurrence or metastasis (if one occurred), or the last follow-up if the patient did not progress. Fig. 2.4.4D shows irrespective of receptor status (n=120), patients with higher centrosome aberrations (>20%) had lower PFS (p<0.0988) than patients with lower centrosome aberrations (<20%). Interestingly, a majority (~72%) of high CA (high-risk) group was TNBC while low CA (low-risk) group was largely non-TNBC (~60%).
Figure 2.4.4 TNBC patients show higher expression of centrosomal and aggressiveness markers.

Ai. Micrographs showing immunohistchemical staining for Aurora-A, Plk4 (centrosomal amplification markers) and Vimentin (breast cancer metastasis markers) in normal and cancer tissue from grade matched TNBC (2170) and non-TNBC (6456) patients. B. Box whisker graph showing significantly higher expression of Vimentin in TNBC samples when compared to non-TNBC samples. C. Immunoblots for normal, TNBC (2170) and non- TNBC (6456) tissue samples. D. Overall survival plot for the patients with higher (>20%) CA (blue) and lower (<20%) CA (red).

2.4.4 Cells with amplified centrosomes show higher migration velocity and net displacement compared to cells with normal centrosomes

To evaluate if extra centrosomes are associated with cell migration, we performed a cell migration assay using time lapse imaging wherein migration of MDA-MB-231 cells (stably transfected with GFP-tagged centriolar protein, centrin) harboring 1 centrosome or >2 centrosomes was observed. Fig. 5... shows representative data for a pair of cells observed by 18h live imaging, where a cell with 1 centrosome migrated from a to a’ (Fig. 2.4.5 Ai) and a cell with 4 centrosomes moved from b to b’ (Fig. 2.4.5Aii). Quantitation of merged time-lapse sequences (10 min apart, collected over 18h) showed higher average net displacement (78%), displacement
rate (55%) and velocity (~2 fold enhancement) of cells with >2 centrosomes compared to cells with 1 centrosome (Fig. 2.4.5Bi,ii,iii). These data strongly suggest that overabundance of centrosomes enhances migratory ability in cancer cells. Having established a strong correlation between extra centrosomes and cell migration in an aggressive breast cancer cell line, we next asked if centrosome amplification directly and independently impacts the migration potential of a mildly aggressive breast cancer cell line, MCF-7. We evaluated if generation of extra centrosomes by genetic means will impact migration and invasion kinetics in breast epithelial cells. To this end, we used MCF-10A cells engineered to facilitate inducible overexpression of wild-type Plk4 or truncated plk4 (1-608, negative control), upon doxycycline treatment for 48h. CA upon treatment was confirmed by immunofluorescence staining for γ-tubulin and change in γ-tubulin levels by western blot in cells overexpressing wild-type Plk4, whereas no amplification was observed in cells overexpressing truncated Plk4. We also observed a significant increase in vimentin levels along with centrosomal proteins upon doxycycline-based induction of centrosome amplification. We then performed a classical wound healing assay to assess the migratory capacity of cells with amplified centrosomes (~80% cells harboring extra centrosomes) as compared to the control cells. We found that cells with CA filled the scratch wound in less than half the time as control cells (18h as compared to 40h). Additionally, an increase in the invasion capacity of wild-type Plk4 overexpressing cells was observed via Boyden chamber assay. Fig. 2.4.5C shows a significantly higher number of cells invaded from the top of the chamber to the bottom of the filter after 48h of incubation.

These data collectively underscore the critical role of centrosomes in facilitating directed cell migration and invasion. These observation lay the experimental foundation that link CA, an important cellular feature of certain cancers to more aggressive phenotypes and provide a
tantalizing possibility that organellar-level differences may serve as a predictor of metastatic risk in breast cancer patients.

![Image](image_url)

**Figure 2.4.5** Cells with amplified centrosomes show higher migration potential.

**Ai, ii** Time lapse images over 18hr of GFP-centrin- MDA-MB-231 cells with one (i/above) versus four (ii/below) centrosomes. Trajectories of 10 cells each were captured over 18hr with 108 images per movie (6 frames/hr). Data was analyzed using velocity3.0 software (Improvision Covery, UK) and average net displacement, displacement rate and velocity measurements were generated for the identified cells with respect to cell centroids. Quantitation of net displacement (Bi) displacement rate (Bii) track velocity (Biii) for cells with 1 and >2 centrosomes are shown in bar graphs. C. Bar graph showing the number of migrated cells in Boyden chamber assay performed with control and Plk4 induced/overexpressed MCF-10 A cells.

2.5 Discussion

A century ago, Theodore Boveri proposed that subtle mitotic errors in dividing cells could be responsible for generating aneuploidy, chromosomal instability and thus tumorigenesis. This pioneering theory originated from his observations that increased number of centrosomes lead to multipolar mitosis and highly aneuploid daughter cells. Since then, several landmark studies have demonstrated that the centrosome plays an important role in establishing cellular polarity, erection of a fusiform spindle apparatus that ensures accurate chromosome segregation.
It is well established now that abnormal number of centrosomes lead to aberrant mitotic divisions and aneuploidy, all of which are frequently observed in many human cancers. In addition, chromosomal instability, originating from generation of aneuploidy over time, has been associated with various prognostic factors in breast cancer. All these studies point towards a strong correlation between centrosome amplification and tumor progression, recurrence and poor survival yet the mechanistic aspects of this relationship have remained elusive. Recently, David Pellman’s laboratory has provided convincing cell-based evidence that centrosome amplification can cause oncogene-like effects to promote cellular invasion in mammary epithelial cells. These findings assert that structural alteration of the cytoskeleton via centrosome amplification confers transformation potential on normal epithelial cells and is directly responsible for tumor initiation and progression. This study substantiates our published hypothesis that cells endowed with extra centrosomes possess cytoskeletal advantages that enhance cell polarization, Golgi-dependent vesicular trafficking, stromal invasion, and other aspects of metastatic progression.

A miscellany of centrosomal defects such as increase in number, size, and atypical structure comprise centrosome amplification. Centrosomal amplification in human cancers entail both structural and numerical aberrations. Structural defects can be due to abnormal centriole structure and/or abnormal amount of pericentriolar material (PCM). While detection of alterations in centriole size is precise using centriole-specific antibody, an increased amount of PCM, detected by a pericentriolar antibody, can present complex ambiguous scenarios. One possibility is increased PCM, making it a ‘true structural defect’. Another possibility is that supernumerary centrosomes that are tightly clustered during interphase may appear as a large centrosome, and thus in fact be a ‘numerical defect’. In several cases, one can notice supernumerary centrosomes with increased PCM, making it both a structural and numerical
defect. These issues render quantitation of centrosomal defects very subjective and dependent on the viewer’s discretion. Therefore, better methods to systematically assess and distinguish centrosome abnormalities are needed. Numerical defects in the form of centrosome amplification have been widely described in human cancer, but not much is known about the structural defects. It is likely that both numerical and structural defects may have distinct implications in the course of tumor progression. In early stage tumors, numerical defects such as centrosome amplification may advantageously serve cancer cells by offering them a means to generate an array of clones that form the basis of intratumor heterogeneity. Once these heterogeneous clones attain desirable karyotypes, we speculate that individual centrosomes tend to cluster and may endow cells with cytoskeletal-derived mechanical advantages for directional migration and that might explain why certain non-invasive lesions transform into highly aggressive tumors with metastatic capabilities while others tend to remain indolent. In addition, it is reasonable to postulate that an increased centrosomal complement will perhaps have an enhanced microtubule nucleation capacity. A higher microtubule density in cancer cells in conjunction with the action of the actin cytoskeleton may provide cells with protrusion capability for faster migration through the ECM. Centrosomes and the microtubules they nucleate together establish the nuclear-centrosomal axis, which defines direction of cell movement, and control post-mitotic Golgi reassembly. Microtubules nucleated on the juxta-centrosomal Golgi serve as tracks for Golgi-derived vesicles carrying proteins essential for leading edge protrusion, and matrix remodeling. Our preliminary data in MDA-MB-231 (where 20-30% of cells display CA) showed that cells with extra centrosomes migrate ~2-fold faster than cells with normal centrosome number. In addition to facilitating faster cellular movement, it is likely that supernumerary centrosomes may impart cells with an enhanced stroma-penetrating capacity through an array of mechanisms including augmentation
of microtubule-dependent MMP secretion. In this study, we have clearly demonstrated a positive correlation between markers of centrosome amplification and vimentin expression in breast cancer patients. These data lay strong grounds for the exploration of centrosomal defects as cellular traits of non-invasive lesions that can potentially determine metastatic risk.

Since majority of breast cancers are characterized by centrosome amplification, the extent, severity and type of amplification can provide insights into the metastatic propensity of certain tumors as opposed to others. Currently-used prognostic indicators (e.g. Ki67) do not predict metastases risk accurately in early-stage tumors. Expensive commercial multigene expression assays such as OncotypeDx provide a “Recurrence Score” and while treatment paths for patients with scores <18 (low-risk) and >31 (high-risk) are relatively unambiguous, 34% of patients with scores between 18-31 lack additional markers to guide optimal treatment decisions. Given that breast cancers harbor amplified centrosomes, a quantitative estimation of centrosomal abnormalities in fine-needle aspirates or paraffin-embedded samples may prove to be a simple, sensitive, and easily quantifiable risk-predicting biomarker.

Precise prognosis suffers challenges owing to various confounding factors with bearing on breast cancer patient outcomes such as race, age, receptor-status, molecular signatures and so on. For instance, among African-American women who develop breast cancer, there is an estimated 20-40% chance of the breast cancer being triple-negative. It is well known that TNBC accounts for ~15% of all breast cancer in the United States. It seems to be more common in (a) young women, (b) women of African ancestry, and (c) individuals with BRCA1 mutations. Cancer recurs in ~30% of early-stage TNBC patients, usually within the first few years after treatment. Further studies are required to understand why premenopausal women and women in some ethnic groups have higher rates of triple-negative breast cancer than other groups of
women, and what mechanisms underlie the aggressive traits of TNBCs. Such studies will be critical in order to device relevant treatment strategies specific for the particular group of patients or tumor subtypes. However, finding suitable targeting pathways in TNBC is proving to be a challenge, particularly because these biologically aggressive tumors do not rely on one dominant pathway, as do ER-positive or HER2-positive tumors. Several studies have led to the realization that TNBC tumors are a very mixed bag and this extensive heterogeneity reported in TNBC provides a strong indication that CA that underlies karyotypic diversity could be the underlying cause of the aggressive nature of this breast cancer subtype. Recent reports focused on identifying the key genetic and molecular features of individual TNBC tumors led to the identification of 7 TNBC subtypes, as follows: basal-like 1 (BL1); basal-like 2 (BL2); immunomodulatory (IM); mesenchymal (M); mesenchymal stem–like (MSL); luminal androgen receptor (LAR); and unstable (UNS). Such a classification based on differential gene and molecular expression is a significant effort in unmasking specific susceptibilities of tumors, with can be targeted for tailored therapies. While the ginormous effort and cost associated with such gene expression based assays limit their feasibility and prompt use in the clinic, non-invasive centrosome-based detection methods (e.g. in fine-needle aspirate cytology) may allow early distinction between indolent and potentially fatal breast cancers.

2.6 References


3. **CAS: AN INNOVATIVE METHOD TO QUANTITATE CENTROSOME AMPLIFICATION**

3.1 **Introduction**

Centrosome amplification (CA) occurs early (even in pre-cancerous and pre-invasive lesions) and may play a very early, causal role in actively driving tumor progression. About 80% of breast cancers exhibit centrosome amplification (CA), a potential indicator of cancer aggressiveness. Centrosomal abnormalities could be structural and/or functional (e.g., altered microtubule-nucleating capacity) and could cause cytoarchitectural distortion and loss of cellular differentiation in tissues. Since centrosomes drive cell polarization and directional migration, which are essential for metastases, differences in centrosome biology may underlie this disparate burden. Although recent research has focused on identification of gene expression signatures in tumors with differing metastatic potential, organelle-level disparities between breast tumors of differing metastatic potential have remained unexplored. Furthermore, a simple and reliable clinically-validated diagnostic test to predict metastatic risk in low-grade tumors has remained elusive. We have developed an innovative first-of-its-kind quantitative method to evaluate the extent of numerical as well as structural CA within tumor samples. Our statistically-validated algorithm defines a total measurable index called Centrosome Amplification Score (CAS_{total}) which includes contributions from both numeral (CAS_{i}) and structural (CAS_{m}) amplification. Since centrosome-nucleated microtubule arrays enable cell polarization and directional migration, a higher CAS_{total} with a bias towards structural abnormalities (enlarged megacentrosomes, high CAS_{m}) may underlie an aggressive disease course. Thus, we hypothesize that breast tumors that have centrosomal overabundance, in particular, enlarged structurally distorted centrosomes with high microtubule nucleating capacity tend to proceed towards
acquisition of a more aggressive disease course. This study aims to transform current research and clinical practice paradigms by 1) interrogating centrosome amplification as a quantifiable “cell biological property” to predict risk of a non-invasive early-stage breast cancer becoming metastatic, 2) defining the previously unrecognized role of amplified centrosomes in promoting metastases, 3) enabling patient stratification for selection of optimal therapy.

3.2 Method

The CAS has two components, CAS$_i$ and CAS$_m$.

CAS$_i$ is an aggregate value of both frequency and severity of **numerical** centrosome amplification, and is scaled relative to the range found in normal somatic tissue.

CAS$_m$ is an aggregate value of both frequency and severity of **structural** centrosome amplification, and is scaled relative to the volume range found in normal somatic tissue.

$$CAS_{total} = CAS_i + CAS_m$$
3.3 Results

3.3.1 Breast tumors from African-American (AA) women show higher centrosome counts and structural aberrations than grade-matched Caucasian women

We employed CAS to quantify CA (numeral and structural) in receptor- and grade-matched tissue from AA (n=65) and Caucasian (n=55) women (Fig. 3.3.1A). Dr. Gabriela Oprea, Pathologist, Grady Hospital (letter attached), provided us these 120 paraffin-embedded breast cancer tissues in a Tissue Microarray (TMA) format to generate this preliminary data. We immunofluorescently stained these samples using antibodies against gamma-tubulin (centrosomal marker, green) and DAPI (nuclear stain, blue) and quantitatively determined iCTRs and mCTRs by examining centrosomes in 10 high power fields ((HPFs, 63X magnification, NA=1.4) and CAS was calculated using formula shown in schematic above. Fig. 3.3.1B shows that CAS was significantly higher in breast tumors derived from AA (mean=8.6) compared to Caucasian women (mean=6.4) (p<0.033) that were grade- and receptor- matched.

3.3.2 Higher centrosomal aberrations are associated with reduced progression-free survival

Next, we evaluated the relationship of higher centosome aberrations (as assessed by CAS) with PFS in breast cancer patients. PFS was calculated as the number of days from diagnosis to the first local recurrence or metastasis (if one occurred), or the last follow-up if the patient did not progress. Irrespective of receptor status (n=60), patients with more centrosomal aberrations (ie., high CAS) had shorter PFS (p<0.0988) than patients with lesser centosome aberrations (ie., low CAS) (Fig. 3.3.1C). Interestingly, a majority (>85%) of high CAS (high-risk) group was AA while low CAS (low-risk) group was mostly Caucasian (>80%).
Figure 3.3.1 Centrosomal disparity between AA and Caucasian women.
A. Immunomicrographs showing higher incidence and extent of amplification in AA than Caucasians (β-tubulin, green). B. Quantitation of CAS values in Caucasians and AA. C. Kaplan-Meir survival curve indicates much poorer progression-free survival of “high CAS” patients (with more centrosomal aberrations) when compared to “low CAS” patients.
4. **INDUCTION OF ROBUST DE NOVO CENTROSOME AMPLIFICATION, HIGH- GRADE SPINDLE MULTIPOLARITY AND METAPHASE CATASTROPHE: A NOVEL CHEMOTHERAPEUTIC APPROACH**¹

4.1 Abstract

Centrosome amplification (CA) and resultant chromosomal instability have long been associated with tumorigenesis. However, exacerbation of CA and relentless centrosome declustering engender robust spindle multipolarity (SM) during mitosis and may induce cell death. Recently, we demonstrated that a noscapinoid member, reduced bromonoscapine, (S)-3-(R)-9-bromo-5-(4,5-dimethoxy-1,3-dihydroisobenzofuran-1-yl)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-[1,3]dioxolo-[4,5-g]isoquinoline (Red-Br-nos), induces reactive oxygen species (ROS)-mediated autophagy and caspase-independent death in prostate cancer PC-3 cells. Herein, we show that Red-Br-nos induces ROS-dependent DNA damage that resulted in high-grade CA and SM in PC-3 cells. Unlike doxorubicin, which causes double-stranded DNA breaks and chronic G2 arrest accompanied by ‘templated’ CA, Red-Br-nos-mediated DNA damage elicits de novo CA during a transient S/G2 stall, followed by checkpoint abrogation and mitotic entry to form aberrant mitotic figures with supernumerary spindle poles. Attenuation of multipolar phenotype in the presence of tiron, a ROS inhibitor, indicated that ROS-mediated DNA damage was partly responsible for driving CA and SM. Although a few cells (~5%) yielded to aberrant cytokinesis following an ‘anaphase catastrophe’, most mitotically arrested cells (~70%) succumbed to ‘metaphase catastrophe,’ which was caspase-independent. This report is the first documentation

¹Parts of this chapter have been published verbatim in *Cell Death and Disease*. 2012 Jul 12;3:e346. doi: 10.1038/cddis.2012.82. as “Induction of robust de novo centrosome amplification, high-grade spindle multipolarity and metaphase catastrophe: A novel chemotherapeutic approach”.

of rapid de novo centrosome formation in the presence of parent centrosome by a noscapinoid family member, which triggers death-inducing SM via a unique mechanism that distinguishes it from other ROS-inducers, conventional DNA-damaging agents, as well as other microtubule-binding drugs.

4.2 Introduction

Cancer cells are often characterized by centrosome amplification (CA) and chromosomal instability. Although supernumerary centrosomes can be tumor-promoting by inducing low-grade aneuploidy, they present a potential for multipolar mitoses that may lead to high-grade, death-inducing aneuploidy. To escape a multipolar configuration, cancer cells have evolved ‘clever’ tactics to suppress multipolarity by pseudo-bipolar centrosome clustering during mitosis. Because cancer cells are inherently vulnerable to induction of CA unlike normal cells, any CA-inducing agent is likely to carry the additional advantage of being cancer cell-specific. An emerging paradigm thus ascribes robust induction of spindle multipolarity (SM) by CA and persistent centrosome declustering as a potentially attractive two-pronged chemotherapeutic approach. Thus, strategies exploiting powerful induction of supernumerary centrosomes are becoming center stage for cancer-selective therapeutic intervention.

Cell-cycle progression is intimately integrated with oscillations in oxygen consumption, energy metabolism and redox state, all of which rely on reactive oxygen species (ROS) levels. Because the array of targets that respond to changes in redox status fluctuate during the cell cycle, ROS levels affect cell fate variably. Centrosomes, also called ‘command centers for cellular control’, have been recently identified as an integration hub of several signaling
pathways, including the DNA-damage response for cell-cycle arrest and repair following ROS-mediated stress.\textsuperscript{10, 11} Several reports suggest existence of a centrosome inactivation checkpoint that utilizes DNA-damage-induced CA to provoke cell death during mitosis, referred to as ‘mitotic catastrophe’.\textsuperscript{10} However, cells refractory to mitotic catastrophe may proceed to yield multiple karyotypically unstable or nonviable daughter cells. This has been recently named ‘anaphase catastrophe,’ a phenomenon that can be pharmacologically induced for selective targeting of cancer cells.\textsuperscript{12}

Continued efforts in our laboratory are focused on expanding a novel class of microtubule (MT)-modulating anticancer agents, noscapinoids, based upon the founding molecule, noscapine.\textsuperscript{13, 14, 15} Unlike conventional tubulin-binding agents, these small molecules gently attenuate MT dynamics without altering the total polymer mass of tubulin.\textsuperscript{6, 16, 17} A novel noscapinoid, reduced bromonoscapine, \((S)-3-(R)-9\text{-bromo}-5-(4,5\text{-dimethoxy}-1,3\text{-dihydroisobenzofuran-1-yl})-4\text{-methoxy-6-methyl}-5,6,7,8\text{-tetrahydro-[1,3]dioxolo-[4,5-g]isoquinoline (hereon referred to as Red-Br-nos), is significantly more potent than noscapine and its extensively studied congener, 9-bromonoscapine (also known by EM011).\textsuperscript{16, 18, 19} Recently, we reported that Red-Br-nos induces mitochondrially driven ROS-dependent autophagic cell death that was caspase-independent in prostate cancer PC-3 cells.\textsuperscript{19} However, key cell-cycle events responsive to ROS induction that can intercede life and death decisions upon Red-Br-nos exposure still remain elusive. Herein, we take the study to the next level by closely investigating outcomes of Red-Br-nos-induced ROS production on cell-cycle phase-specific events and analyzing how that translates into cell death in a caspase-refractory setting in prostate cancer cells.
Our data demonstrate that Red-Br-nos induces ROS-dependent DNA damage, which causes de novo CA associated with increased cdk2 activity and enhanced polo-like kinase-4 (PLK4) expression. Despite activation of DNA-damage-sensitive kinases, cells were transiently arrested in S or G2 phases and bypassed the DNA-damage checkpoint to enter mitosis. Induction of CA during interphase preceded generation of SM during a stalled mitosis, which was responsive to ROS-mediated DNA damage. This is the first report to identify induction of CA and consequent SM, which promotes mitotic death, in particular ‘metaphase catastrophe’, as the modus operandi for a member of the MT-modulating noscapine family.

4.3 Materials and Methods

4.3.1 Cell lines, culture and drug treatment:

PC-3 cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Red-Br-nos was synthesized from noscapine as described previously. Stock solutions of 50 mM were prepared in DMSO and kept frozen at −20 °C until use. All Red-Br-nos treatments were done at 10 μM concentration for the indicated time points. Tiron was used at 1 mM concentration.

4.3.2 Kinase activity assay:

To examine cdk2 kinase activity, cdk2 antibody was used to selectively immunoprecipitate cdk2 from vehicle- and Red-Br-nos-treated PC-3 cell lysates. The resulting immunoprecipitate was incubated with pure histone-H3 protein in the presence of ATP and kinase buffer. The kinase assay reaction allowed immunoprecipitated cdk2 to phosphorylate histone-H3 in vitro, the extent of which was measured by immunoblotting using phosphohistone-
H3 antibody from Cell Signaling (Beverly, MA, USA). Histone-H3 protein was from Millipore (Billerica, MA, USA) and ATP was from Cell Signaling.

**4.3.3 Immunofluorescence microscopy, cell-cycle analysis and immunoblotting:**

Cells were cultured to ~70% confluence and medium was replaced with fresh medium containing either vehicle (0.1% DMSO) or 10 μM Red-Br-nos for the noted times, followed by processing for immunofluorescence microscopy, flow cytometry or immunoblotting as described previously. Mitotic index was determined by using mitotic protein monoclonal-2 (MPM-2) antibody from Cell Signaling as described previously. Antibodies against γ-tubulin, α-tubulin and β-actin were from Sigma (St. Louis, MO, USA). Anti-BubR1 antibody was from BD Biosciences (Pharmingen, San Jose, CA, USA) and human anti-centromere ACA antibody for kinetochore staining was from Antibodies, Inc. (Davis, CA, USA). Antibodies for γ-H2AX, PLK4, p-bcl2, p-ATR and p-chk1 were from Cell Signaling. Cenexin antibody was a generous gift from Dr. Stephen Doxsey, University of Massachusetts (Worcester, MA, USA). Alexa 488- or 555- conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Detailed analysis of γ-H2AX immunofluorescence signal intensity was performed utilizing Metamorph analysis software (Molecular Devices, Sunnyvale, CA, USA). The final output for both vehicle- and Red-Br-nos-treated cells was the integrated intensity based on total stained area and staining intensity at individual pixels. Cell-cycle profiling was done on LSRFortessa flow-cytometer (BD Biosciences) and analyzed using Flowjo software (Tree Star, Ashland, OR, USA).
4.3.4 **Database search method for the numerical analysis of centrioles:**

Observations of the number of ‘templated’ *versus de novo* centrioles were recorded in a simple database in the following manner. Each cell studied was allocated a unique identification number (cell ID). For each cell ID, there were zero or more mother centrioles present. Each was given a unique identification number (mother ID) for that cell and labeled M1, M2 and so on. For each mother ID, the number of associated daughter centrioles was recorded. Additionally, zero or more isolated *de novo* groups of centrioles may be present for the cell. For each such group present, a unique identification number was allocated and labeled D1, D2 and so on. For each *de novo* group ID, the number of associated daughters was recorded. In a similar manner, any daughters shared by two mothers (there was no instance of more than two mothers sharing daughters) were recorded with labels S1, S2 and so on. Complete records for two successive cells as it appeared in the database are represented by data in Supplementary Table 1. Two databases were created by reading a comma-separated list of records into a simple program written in the Python programming language, specifically designed for these data. One database contained 150 cells of *TYPE_A_Rd-Br-nos* and the other contained 150 cells of *TYPE_B_Doxo*. The databases were each searched for a total of four queries as discussed in the Results section as well as in the legend to Figure 3.

4.3.5 **Statistical analysis:**

All experiments were repeated three times. Data were expressed as mean±S.D. Statistical analysis was performed using Student’s *t*-test. The criterion for statistical significance was *P*<0.05.
4.4 Results

4.4.1 Red-Br-nos-induced ROS-dependent genotoxic stress activates a DNA-damage checkpoint response

Several studies suggest that ROS may directly modulate cell-cycle progression. Depending upon the magnitude and duration of ROS exposure, activation of growth-factor-stimulated signaling cascades may promote cell-cycle progression upon low levels of ROS exposure or cause growth arrest upon prolonged ROS exposure. Oxidative damage produced by intracellular ROS often results in DNA-based modifications and single- and double-strand breaks. This may alert a ‘salvage’ strategy like checkpoint surveillance to stall the cell cycle or a ‘disposal’ mechanism like apoptosis to eliminate irreparable cells. We have recently demonstrated that Red-Br-nos (25 μM) causes ROS induction in PC-3 cells as well as at a lower dose of 10 μM (data not shown). Thus, we first asked whether Red-Br-nos-induced ROS causes DNA damage in PC-3 cells. To this end, we microscopically examined drug-treated cells over time for the presence of foci of γ-H2AX, the phosphorylated form of histone H2AX that forms around sites of DNA breakage. Phosphorylation of H2AX (γ-H2AX) is an early chromatin modification and a sensitive marker for double-strand breaks. We observed γ-H2AX foci as early as 3 h post Red-Br-nos treatment and a peak at 18 h, demonstrating a time-dependent increase in DNA damage throughout interphase (Fig. 4.4.1ai). On the contrary, vehicle-treated control cells lacked γ-H2AX foci, indicating that drug exposure induced DNA-strand breaks. There was not only a time-dependent increase in number of cells with γ-H2AX foci but also an increase in the number and intensity of γ-H2AX foci per cell, indicating a rise in the extent and severity of DNA damage inflicted by Red-Br-nos over time (Fig. 4.4.1aii and iii).
Figure 4.4.1 Red-Br-nos triggers ROS-mediated DNA-damage checkpoint response in PC-3 cells.

(ai) Representative immunofluorescence confocal micrographs showing emergence of γ-H2AX foci indicative of DNA damage upon treatment with Red-Br-nos (10 μM) over time. Panels show DNA (DAPI), γ-H2AX (green) and MTs (red). (aii and iii) Bar-graph quantitation of number and intensity, respectively, of γ-H2AX foci per cell over time. (b) Immunoblot analysis of γ-H2AX and DNA-damage checkpoint response markers, p-ATR and p-chk1, at the noted time points. β-actin was used as the loading control. (ci) Attenuation of ROS upon a 4-h tiron treatment prior to Red-Br-nos exposure for 12 h showed a marked reduction in the number of cells harboring γ-H2AX foci (green) as compared with drug treatment alone. (cii) Immunoblot showing significant reduction in γ-H2AX expression when tiron was co-treated with drug compared with drug alone. Scale bar=5 μm
We next asked if γ-H2AX foci recruit additional proteins involved in the DNA-damage checkpoint response. The serine/threonine protein kinases ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) are transducers of DNA-damage checkpoint responses. Although ATM responds to IR-induced double-strand breaks and activates chk2, ATR responds to DNA-damaging agents such as UV light and activates chk1. Activated chk2 and chk1 in turn inactivate cdc25C phosphatase by phosphorylation, which leads to a G2/M arrest. Although ATM levels remained unaffected by Red-Br-nos (data not shown), we found enhanced p-ATR expression, which perhaps increased chk1 activation, constituting a DNA-damage checkpoint response to Red-Br-nos treatment (Fig. 4.4.1b).

Having identified Red-Br-nos-induced DNA-strand breaks, we next addressed whether induction of DNA damage was ROS-dependent. To this end, PC-3 cells were pretreated for 4 h with tiron, a ROS scavenger, and γ-H2AX foci were examined after 12 h of Red-Br-nos exposure (Fig. 4.4.1ci). Attenuation of ROS levels by tiron decreased the number of cells with γ-H2AX foci by ~88% following 12 h Red-Br-nos treatment (Fig 4.4.1ci). This contrasted with Red-Br-nos treatment alone that caused an induction (~90%) of γ-H2AX foci-containing cells (data not shown). Attenuation of Red-Br-nos induced γ-H2AX levels upon tiron treatment was also confirmed by immunoblotting (Fig. 4.4.1cii). However, we also saw low levels of γ-H2AX expression upon tiron treatment alone. Although several studies including ours have shown that tiron acts as a ROS scavenger, there are reports that high-concentration tiron (>0.5 mM) induces ROS-independent DNA damage. In our study, we used tiron at 1 mM, and this concentration likely resulted in low-level ROS-independent DNA damage.
4.4.2 Red-Br-nos-induced DNA damage mediates high-grade CA

Consonant with its protective function, the DNA-damage response may serve as an anticancer barrier in early human tumorigenesis. One important mediator of the DNA-damage response, chk1, negatively regulates G2/M transition via its centrosomal localization, emphasizing a role for centrosomes in the DNA-damage response. Centrosomes may serve as spatiotemporal organizers that juxtapose DNA checkpoint players in a defined manner. Alternatively, if the centrosome cycle is regulated by DNA damage, centrosomes might serve as effectors of DNA-damage response, resulting in apoptosis-inducing centrosome inactivation or fragmentation. Yet another possibility is that activation of the DNA-damage response might trigger CA. To investigate effects of drug-induced DNA damage on centrosomes, we immunostained PC-3 cells with γ-tubulin, a centrosome-specific marker. We observed several γ-tubulin spots ranging from 2 to 11 per cell as early as 12 h post drug treatment (Fig. 4.4.2ai and ii). Intriguingly, we observed that ~70% of drug-treated cells showed an abnormal number of centrosomes (n>2). Although γ-tubulin is a centrosomal protein, its pericentriolar material (PCM) distribution is well known. Thus, to rule out centrosome fragmentation, we immunostained Red-Br-nos-treated cells with centrin-2, a specific centriolar marker (Fig. 4.4.2bi and ii). Immunofluorescence confocal micrographs revealed several centrin spots in cells treated with Red-Br-nos for 12 h, suggesting that drug-treated cells exhibit ‘true’ CA involving generation of ‘real’ centrioles rather than just PCM fragmentation. These findings were further supported by our observations of time-lapse imaged, live MDA-MB-231 cells stably transfected with GFP-centrin that were treated with 10 μM Red-Br-nos for 9 h. We found that drug treatment induced extensive centriole amplification within 3 h (data not shown) unlike vehicle-treated control MDA-MB-231 cells (data not shown).
Several mitotic kinases, including PLK4 and Aurora A, have been shown to regulate centrosome-duplication events.\textsuperscript{28, 29} Importantly, PLK4 is not only implicated in centriole
overduplication\textsuperscript{30} but also has been shown to deposit centriole precursor material in a rosette-like arrangement around maternal centrioles.\textsuperscript{31} Thus, we wanted to determine whether Red-Br-nos-induced CA was accompanied by enhanced PLK4 expression levels in PC-3 cells. Immunofluorescence as well as immunoblotting data confirmed the increase in PLK4 expression upon drug treatment (\textbf{Fig. 4.4.2c and d}). Interestingly, drug treatment increased cdk2 activity, as seen by enhanced expression of phosphohistone-H3 (\textbf{Fig. 4.4.2e}). Pretreatment with tiron before drug exposure attenuated the number of cells harboring multiple centrosomes, suggesting that this CA event is responsive to and downstream of DNA damage induced by ROS (\textbf{Fig. 4.4.2fi and ii}). These data suggest that Red-Br-nos treatment causes upregulation of the centriolar biogenesis machinery and thus creates cytoplasmic conditions conducive to rapid centriolar assembly.

\textbf{4.4.3 Red-Br-nos induces de novo CA}

Having uncovered that Red-Br-nos induces robust CA, we focused our attention on deciphering the basis of this brisk CA. Given the increased proportion of cells with extensive CA within 9 h of drug treatment, ‘templated’ centrosome overduplication seemed an unlikely explanation. Thus, we sought to determine whether the amplified centrioles emerged \textit{de novo} or through a ‘templated’ mechanism by centrosome overduplication. Because centriole maturation is an important functional property of the centriole, we immunostained drug-treated cells with cenexin, a specific marker for mature (mother) centrioles. The various mother-daughter combinations in both Red-Br-nos- and doxorubicin-treated PC-3 cells were visually scored using immunofluorescence microscopy (\textbf{Fig. 4.4.3}). We did not observe a significant number of ‘rosette-like’ centrally positioned mother centrioles, and daughter centrioles appeared scattered throughout the cytosol far from mothers. Thus, we favor the idea that Red-Br-nos-induced CA is
more likely to be an exceptional case of *de novo* centrosomal assembly occurring in presence of parent centrioles. This may be due to local accumulation of high concentration of centriolar material rather than concurrent formation of multiple daughters at the mother centriole. However, we found a few cells (~5%) wherein a single maternal centriole concurrently generated multiple daughter centrioles, as seen by a ‘rosette-like’ pattern of one cenexin-positive mother centriole surrounded by several centrin-positive daughters (Figure 3-4Ai, panel bv). Overall, a significant increase in cells with more than one daughter per maternal centriole was observed. Red-Br-nos-induced *de novo* centriolar assembly is particularly intriguing given that nocodazole, which disassembles MTs, actually prevents *de novo* centriole formation.\textsuperscript{32} This may be ascribed to the ‘gentler’ MT-modulating effects of Red-Br-nos, which does not alter the monomer/polymer ratio of tubulin even at high concentrations.\textsuperscript{16} Although Red-Br-nos binds soluble tubulin as evidenced by kinetic quenching of intrinsic tryptophan fluorescence of tubulin (data not shown), a concentration of up to 200 $\mu$M did not induce significant structural damage to tubulin as indicated by an absence of change in 1-anilinonaphthalene-8-sulfonic acid-tubulin fluorescence upon addition of Red-Br-nos (data not shown). Instead, it seems that Red-Br-nos binds ‘gently’ to tubulin, which results in attenuated MT dynamics rather than drastic de- or overpolymerization. This attribute perhaps distinguishes Red-Br-nos from other MT-interfering drugs that exert structural damage to MTs. The CA induced by Red-Br-nos also distinguishes it from other conventional chemotherapeutics that cause CA. For instance, doxorubicin, a DNA-intercalating drug, induces CA with a nominal daughter (cenexin-negative) to mother (cenexin-positive) ratio (Fig. 4.4.4B). This observation suggests that CA occurs by several rounds of duplication resulting in numerous mother centrioles with extra daughter centrioles, perhaps during an enhanced cell-cycle arrest. Because daughter centrioles are held close to their mother
by a linker\textsuperscript{33} unlike \textit{de novo} centrioles, scoring was based on physical distance between mother and daughter centrioles for all observed patterns (Fig. 4.4.3). A physical distance of ≤0.2 μm between mother and daughter centriole was categorized as ‘templated’, whereas a distance >0.2 μm was considered \textit{de novo}. Visual observations of the number of ‘templated’ \textit{versus de novo} centrioles, in 150 cells each, were recorded in a simple database. This allowed us to detect trends and statistically quantify and compare numerical centriole aberrations in Red-Br-nos- and doxorubicin-treated cells. Databases for both Red-Br-nos and doxorubicin were queried to address number of cells containing: (a) no \textit{de novo} daughters; (b) a daughter: mother ratio ≥2; (c) ≤2 mothers (i.e., cells with no instance of CA resulting from several rounds of duplication); and (d) at least one mother with >1 daughter (i.e., cells with at least one instance of ‘templated’ overduplication). Our first database query (a) to uncover the percentage of cells with an absence of \textit{de novo} centrioles yielded a significantly higher proportion of cells devoid of \textit{de novo} centrioles upon doxorubicin treatment compared with Red-Br-nos (126 cells with no \textit{de novo} centrioles upon doxorubicin treatment compared with only 12 in Red-Br-nos-treated cells, out of 150 cells). Our next query (b) revealed a significant number of cells bearing a very high ratio of daughter:mother (≥2) upon Red-Br-nos treatment as compared with doxorubicin. A search for cells having ≤2 mother centrioles revealed that there were more instances of CA resulting from several rounds of centrosome-duplication cycle following doxorubicin \textit{versus} Red-Br-nos treatment. Lastly, querying for cells harboring at least one mother associated with more than one daughter (daughters at a distance closer than 0.2 μm) yielded a higher number of cells showing ‘templated’ overduplication for doxorubicin (107/150 doxorubicin-treated cells \textit{versus} 68/150 Red-Br-nos-treated cells) These analyses suggest a spatially more restricted or ‘templated’ pattern of centriole overduplication in case of doxorubicin, which is compromised by Red-Br-
nos (Fig. 4.4.4Ci). We further compared the extent of \textit{de novo} centriole formation in cells exhibiting ‘normal-like’ CA consequent to multiple rounds of duplication with ‘templated’ CA events. The number of \textit{de novo} centrioles was counted in the output of queries (c) and (d) for both Red-Br-nos- and doxorubicin-treated cells. The average number of \textit{de novo} centrioles was plotted as shown in Fig. 4.4.4Cii and iii. We found that the average number of \textit{de novo} centrioles was much higher with Red-Br-nos, irrespective of ‘templated’ overduplication. Collectively, these data suggest the occurrence of more extensive \textit{de novo} centriolar formation in Red-Br-nos-treated cells compared with doxorubicin.
Figure 4.4.3 Schematic illustration of three representative cells depicting all the various mother-daughter centriole combinations that were observed upon Red-Br-nos or doxorubicin treatment.

Cell I shows two mother centrioles (green), namely M1 and M2, and six daughter centrioles (red). M1 has one closely associated daughter (<0.2 μm) and represents a ‘normal’ centrosome or is a result of a normal duplication event. M2 has three daughters lying in close vicinity (~0.2 μm) representing ‘templated’ overduplication. D1 refers to a de novo-formed pair of centrioles, because they are not associated with any mother (> 0.2 μm). Cell II shows two mother centrioles (green) and seven daughter centrioles (red). S1 represents a ‘shared’ situation where a single daughter is shared between two mothers. We cannot exclude the possibility that the ‘templated’ or de novo-formed centrioles later mature to form mothers. D2 and D3 represent two separate clusters of de novo centrioles separated by a distance of >0.2 μm. Cell III shows three mother centrioles (green) and seven daughter centrioles (red). S2 represents another shared situation where two daughters are shared between two mother centrioles. This situation could arise because of two successive rounds of duplication or may even represent a normal G2 situation.
M3 represents a lone mother. D4–D8 represent de novo centrioles lying far apart from each other (>0.2 μm).

4.4.4 Red-Br-nos abrogates DNA-damage-induced G2 checkpoint leading to mitotic entry

In normal cells, S and G2/M checkpoints prevent cells with incompletely replicated or damaged DNA from entering mitosis. However, cancer cells possess compromised checkpoints and cell-cycle arrest due to DNA damage may be weak and relatively easy to breach. Anticancer drugs that exacerbate DNA damage and inactivate the G2 checkpoint to induce apoptosis can capitalize on this Achilles’ heel of cancer cells. Thus, we next explored whether PC-3 cells that suffered DNA damage upon Red-Br-nos treatment stalled in S or G2 phase or entered mitosis. We performed a flow-cytometric analysis to investigate how Red-Br-nos affects cell-cycle phases over time. We found that Red-Br-nos did not induce obvious S or G2 arrest, instead, cells slowly progressed through S and G2 and entered mitosis with damaged DNA (Fig. 4.4.4Di and ii). About 25% of PC-3 cells were in mitosis as early as 12 h, and mitotic index peaked at 24 h post treatment. This suggested that Red-Br-nos efficiently mediated abrogation of G2/M checkpoint, causing cells to enter mitosis despite compromised DNA integrity. Moreover, Red-Br-nos induced de novo centrosomes appeared functionally competent as MT-organizing centers because they efficiently orchestrated multipolar spindle assembly.
Figure 4.4.4 Unlike doxorubicin, Red-Br-nos causes de novo centriole formation and mitotic arrest in PC-3 cells.

(Ai) Immunofluorescence confocal micrographs showing various permutations of centrin (daughter) and cenexin (mother) dots in Red-Br-nos-treated (10 μM for 18 h) PC-3 cells. Centrin is shown in red, cenexin in green and DNA in blue. (Aii) Three-dimensional bar-graph plot representing the percent cell population with specified patterns of centrin and cenexin dots. (b) Confocal immunomicrographs showing cells with various permutations and combinations of centrin (daughter) and cenexin (mother) dots in doxorubicin-treated (10 μM for 18 h) PC-3 cells. Centrin is shown in red, cenexin in green and DNA in blue. (Ci) Bar graph depicting the outcomes of our database queries on cells treated with either Red-Br-nos (10 μM) or doxorubicin (10 μM) for 18 h. (a), (b), (c) and (d) refer to the database search queries. (a) is the number of cells with an absence of de novo centrioles, (b) represents the number of cells where ratio of daughters to mothers is ≥2, (c) represents cells with ≤2 mothers, signifying cells lacking amplification due to several rounds of duplication and (d) represents cells with at least one mother associated with more than one spatially close daughter. A total of 150 cells were analyzed in each case. (Cii) The output of query (c) that resulted in number of cells with at least one instance of ‘templated’ amplification upon multiple rounds of duplication was further
analyzed for average number of *de novo* centrioles as shown in bar graph. (Ciii) The output of query (d) resulted in the number of cells exhibiting ‘templated’ duplication that were further analyzed for number of *de novo* centrioles as plotted in bar graph. (Di and Ei) Three-dimensional DNA histograms representing cell-cycle kinetics of PC-3 cells treated with Red-Br-nos (10 μM) or doxorubicin (10 μM), respectively. The X-axis shows DNA amounts representing different cell-cycle phases, the Y-axis shows the number of cells containing that amount of DNA and the Z-axis shows the duration of treatment. (Dii and Eii) Corresponding dual-color dot plots showing the proportion of mitotic cells (MPM-2-positive) as opposed to G2 cells (MPM-2-negative). Scale bar=5 μm.

For the cell-cycle data as well, we based our comparisons of Red-Br-nos to doxorubicin. Unlike Red-Br-nos, doxorubicin-treated PC-3 cells showed a chronic S/G2 arrest at 12 h and the cells stayed in G2 for over 24 h (Fig. 4.4.Ei and ii). During this durable S/G2 arrest, rampant CA became evident. Interestingly, doxorubicin-treated cells with extra centrosomes did not show a high ratio of centrin to cenexin spots. In addition, daughter centrioles appeared closely associated with mother centrioles. These observations were more consonant with multiple cycles of ‘templated’ duplication rather than *de novo* centrosome formation. Intriguingly, doxorubicin-treated cells were chronically ‘stuck’ in G2 and failed to enter mitosis even 28 h post treatment. This phenotype stood in stark contrast to that of Red-Br-nos-treated cells, which were transiently arrested in G2 and abrogated the G2/M checkpoint to enter mitosis as early as 12 h post treatment. Taken together, these data suggest that Red-Br-nos-induced CA occurs via *de novo* biogenesis that differs qualitatively from ‘templated’ centriole duplication observed following doxorubicin exposure.

4.4.5 **CA induces rampant mitotic SM by persistent centrosome declustering**

We next quantitated the number of centrosomes per pole in mitotic cells upon Red-Br-nos treatment using immunofluorescence confocal microscopy. In control cells, all cell-cycle stages appeared normal and mitotic cells exhibited typical bipolar spindles at the expected frequency (data not shown). However, 18 h Red-Br-nos-treated cells showed a preponderance of
multipolar spindles with defects in chromosome congression to metaphase plate (Fig. 4.4.5a). Quantitation of unipolar, bipolar, tripolar, tetrapolar and multipolar ($n>4, n=$number of spindle poles per cell) spindles is shown in Fig. 4.4.5b. Intriguingly, we found that the multipolar phenotype progressively increased over time (data not shown), indicating persistent centrosome declustering. Most spindle poles in a multipolar configuration showed presence of ‘real’ centrioles (~80%, $n=200$), confirming robust CA.
Figure 4.4.5 Red-Br-nos induces high-grade SM in PC-3 cells.
Red-Br-nos induces high-grade SM in PC-3 cells. (a) Immunofluorescence confocal micrographs of PC-3 cells ‘stuck’ in mitosis upon treatment with Red-Br-nos (10 μM) for 18 h. Panels show γ-
tubulin in green, MTs in red and DNA in blue (DAPI). (b) Pie-chart quantitation of the proportion of cells exhibiting specified spindle polarity. The category classified as ‘other’ predominantly includes bipolar mitotic cells or cells exhibiting aneuploidy and ‘mitotic catastrophe’. (c) ROS inhibition by tiron treatment preceding Red-Br-nos treatment (10 μM) for 24 h showed reduced multipolarity as compared with Red-Br-nos treatment alone. Microtubules are shown in red and DNA in blue. (d) PC-3 cells pretreated with cytochalasin D (1 μM) for 4 h and then treated with Red-Br-nos for 18 h showed enhanced spindle-pole amplification as compared with only Red-Br-nos treatment (10 μM) for 18 h. Actin is shown in red, MTs in green and DNA in blue. Scale bar=5 μm.

Having observed ROS-dependent DNA damage that perhaps resulted in CA-driven SM, we were curious to determine whether attenuation of ROS levels affected the severity of spindle-pole amplification during mitosis. To this end, we pretreated cells with tiron for 4 h before Red-Br-nos exposure for 18 h. Our results showed that attenuation of ROS levels with tiron reduced the multipolar phenotype in cells (Fig. 4.4c), perhaps due to a decline in the severity of ROS-induced DNA damage that translated into CA. However, for reasons yet obscure, we also observed an overall decrease in number of mitotic cells with tiron treatment. The mitotic index was assessed using MPM-2, a mitosis-specific marker in a dual-color flow-cytometric experiment. MPM-2-negative cells with 4N DNA were considered as G2, whereas MPM-2-positive/4N DNA cells were read as mitotic. The mitotic population in Red-Br-nos-treated cells at 24 h was ~60%, which dropped to ~10% in 4 h tiron-pretreated cells that were drug-treated for 24 h (data not shown).

We next examined whether formation of multipolar spindles depended on cytoskeletal actin. To this end, cells were treated with cytochalasin D for 4 h followed by a 12-h Red-Br-nos exposure. We found that multipolar spindles with ‘real’ centrioles were retained in presence of cytochalasin D, suggesting that SM was independent of actin filaments (Fig. 4.4d). Surprisingly, cytochalasin treatment increased number of spindle poles. We speculate that
disruption of actin-based centrosome clustering mechanisms (involving interactions of astral MTs with cortical actin) may have enhanced centrosome declustering.\textsuperscript{35,36}

### 4.4.6 Red-Br-nos activates spindle-assembly checkpoint (SAC) and induces ‘metaphase catastrophe’

Having identified rampant spindle-pole amplification upon Red-Br-nos treatment, we wondered if MTs emanating from poles were able to attach properly to kinetochores. Essentially, establishment of optimal sister kinetochore tension is necessary to silence the ‘wait anaphase’ signal of the SAC. As expected, red kinetochore dots across sister kinetochores did not ‘line up’ owing to the presence of aberrant spindle morphology (Fig. 4.4.6a). These unattached or misaligned kinetochores may underlie SAC activation as seen by intense BubR1 staining in drug-treated cells (Figure 3-6b). This observation suggests that lack of tension and/or existence of aberrant kinetochore-MT attachments underlie SAC activation.
Figure 4.4.6 Red-Br-nos activates the SAC and induces 'metaphase catastrophe'.
Red-Br-nos activates the SAC and induces ‘metaphase catastrophe’. (a) Immunomicrographs showing PC-3 cells treated with vehicle or Red-Br-nos (10 μM) for 18 h and stained for kinetochores with CREST (red), MTs with α-tubulin (green) and DNA with DAPI (blue). (b) Panels show BuBR1- (green), actin- (red) and DNA- (blue) stained PC-3 cells treated with vehicle (top panel) or Red-Br-nos (lower panel) for 9 h. (c) Immunofluorescence confocal micrographs representing ‘mitotic catastrophe’ upon Red-Br-nos treatment at the specified time points. Multipolar cells with membrane blebs or protrusions rich in α-tubulin were indicative of cells dying following an unsuccessful metaphase. γ-tubulin is shown in green, α-tubulin in red and DNA in blue. Scale bar=10 μm.
As the aberrant multipolar configuration of cells cannot exist indefinitely, we next evaluated the long-term fate of Red-Br-nos-treated mitotically arrested cells. We determined whether mitotic cells with multipolar spindles directly succumb to cell death, which we refer to as ’metaphase catastrophe,’ or progress to an abnormal anaphase and proceed through aberrant cytokinesis to result in multiple daughters, recently referred to as ‘anaphase catastrophe’. Our immunofluorescence microscopy data showed that a large percentage of mitotic cells (~70%) eventually underwent metaphase catastrophe (Fig. 4.4.6c). Protrusions of tubulin-rich cellular membrane were evident and indicated commencement of apoptosis, perhaps marking a ‘point of no return’. We propose that the process of ‘metaphase catastrophe’ is distinct from ‘anaphase catastrophe’. We found that metaphase cell death was accompanied by extensive membrane blebbing, which was not suppressed by z-vad-fmk, suggesting caspase-independent apoptosis (data not shown). However, a few ‘metaphase-catastrophe refractory’ cells underwent aberrant anaphase to yield ~5% incidence of ‘anaphase catastrophe’. Such cells progressed through mitosis with multiple lobes followed by a complete or incomplete cytokinesis.

4.5 Discussion

CA has long been considered as a hallmark of cancer. Although CA allows maintenance of ‘optimal’ aneuploidy to facilitate tumorigenesis by selection of karyotypes that offer growth advantage, exacerbation of CA may be detrimental to cancer cell survival, thus serving as a favorable chemotherapeutic approach. Essentially, robust CA generates aberrant mitotic spindles with chromosomes migrating to numerous poles, hence causing aneuploidy. Depending upon the degree of aneuploidy, it may be tumor- or death-promoting. Our current study demonstrates for the first time a link between Red-Br-nos-induced early ROS production and DNA damage with
CA-induced SM, which formed the basis of substantial cell death via ‘metaphase catastrophe’ (Fig. 4.5.1).

Figure 4.5.1 Schematic illustration of a proposed model depicting the progression of events upon induction of high ROS levels by Red-Br-nos.
Schematic illustration of a proposed model depicting the progression of events upon induction of high ROS levels by Red-Br-nos. High-grade DNA damage results in transient S/G2 arrest (depicted in yellow/orange) followed by a chronic mitotic arrest (depicted in red). Accumulation of S-phase-specific cyclins/cdk5 results in accrual of PCM components in the vicinity of an already existing and ‘ready to duplicate’ centrosome. CA, predominantly de novo centriole formation along with some degree of ‘templated’ overduplication, occurs during the transient S/G2 arrest, which then translates into excessively multipolar phenotypes during a stalled mitosis. Majority of the arrested multipolar cells succumb to ‘metaphase catastrophe’ due to the chaos arising from multiple insults the cells have suffered including irreparable DNA damage, aberrant kinetochore-MT attachments and spindle multipolarity.

Several reports detail four accepted models of CA etiology,40 including CA resulting from centrosome-nuclear cycle uncoupling,41, 42 cytokinesis failure,43 cell–cell fusion,44 and DNA damage (which actually may simply represent centrosome fragmentation).27 Our data demonstrate that Red-Br-nos-induced CA begins in S phase and continues robustly during the ephemeral G2 arrest. Finally, cells override the G2 checkpoint and enter mitosis in which they succumb to ‘metaphase catastrophe’. Although ATR activation is known to promote recovery of collapsed replication forks, and thus should result in considerable S/G2-phase arrest, this does not seem to be the case. It has been shown that ATR-induced S-phase arrest acts mainly through the p53-dependent pathway.45, 46 Because PC-3 cells are p53-negative,47 despite presence of extensive and severe ROS-induced DNA damage, flow-cytometric analysis indicated only a transient S-phase arrest, suggesting that the observed CA was not accompanied by prolonged S-phase stall. Furthermore, Red-Br-nos caused a loss of the strict correlation between number of centrioles and ploidy, which was clearly not a consequence of failed cytokinesis because a majority of treated cells perished much earlier, that is, in metaphase. Thus, our observations supplement current models by adding a novel mode of CA. This is in addition to our previous observations that showed autophagy (as seen by LC3-II) induction in PC-3 cells treated with Red-Br-nos at 25 μM19 and even lower doses of 10 μM (data not shown). These data collectively
suggest the concurrent induction of ROS-mediated CA and the autophagic pathways that lead to cell death.

Induction of CA by Red-Br-nos shows the following distinctive characteristics: (a) generation of 3–8 centrosomes per cell, (b) ratio of daughter to mother centrioles >1 (i.e., greater than expected for ‘templated’ duplication) and (c) rapid and efficient production of several centrioles in parallel within 9–12 h of drug exposure. Normally, in each individual cell cycle, only one new centrosome is duplicated, close to the preexisting organelle via a semi-conservative ‘templated’ mechanism. In contrast, the canonical description of de novo centriole formation encompasses two key features: (1) it occurs in the absence of preexisting centrioles and (2) the process is slower than normal centriole duplication. Recently, though, the line demarcating normal duplication, ‘templated’ overduplication and de novo centriole formation has blurred. There is speculation ablaze that normal procentriole formation is potentially a type of de novo centriole assembly, only restricted spatially and numerically and hence more tightly regulated. This regulation is orchestrated by the mother centriole that enriches the PCM with centriole biogenesis factors and concentrates the PCM to define the number as well as the proximity of emerging procentrioles. This configuration is then ‘locked’ by linker proteins between mother and procentriole, thus suppressing formation of superfluous daughter centrioles. There are some examples of de novo centrosome formation in nature, for example, in clam zygotes, mice, and rabbit blastomeres. These instances occur in the absence of a preexisting organelle and early in development, when large reserves of maternal products for centriole formation are stored in the oocyte. Also, de novo formation of centrioles occurs exclusively in S phase in Chlamydomonas, and the duplication efficiency is only half that of ‘templated’ assembly. A seminal study demonstrating de novo centrosome formation utilized S-phase-arrested cells in...
which the existing centrosome was destroyed by laser ablation, bolstering the notion that \textit{de novo} centriole formation can only occur in the absence of the parent centrosome.\textsuperscript{32} In this study, \textit{de novo} induction of centriole biogenesis was a slow process beginning only about 5–8 h after centrosome ablation and requiring almost 24 h for completion.\textsuperscript{32}

We found that Red-Br-nos treatment causes rapid and simultaneous production of multiple centrosomes scattered throughout the cytoplasm in the presence of the original centrosome. Evidently, the mother centrosome is failing to spatially restrict procentriole formation to its vicinity and thus cannot limit the number of new procentrioles to one per mother. This could be analogous to having no mother centriole at all (a canonical characteristic of \textit{de novo} formation). We believe that by causing a surge in levels of structural or regulatory components (PLK4 and cdk2) of the centriolar biogenesis machinery that are normally rate-limiting, Red-Br-nos pushes the centrosome-duplication machinery into overdrive. The unique intracellular environment thus created would account for the accelerated and efficient production of centrioles throughout the cytoplasm. This intracellular state is different from that in studies involving laser centrosome ablation and may account for the unusual briskness in \textit{de novo} centriole formation upon Red-Br-nos treatment. PLK4 overexpression causes concomitant formation of multiple daughter centrioles in a ‘rosette’ configuration around a single maternal centriole,\textsuperscript{31, 55} which we observed in rare instances. Thus, both the pathways of \textit{de novo} formation and (albeit to a much smaller extent) ‘templated’ overduplication are activated upon Red-Br-nos treatment leading to a ‘centrosome overload’. Our observations thus concur with the diminishing lines separating the different CA pathways and support the hypothesis that the ‘templated’ mechanism for centriole replication is needed, not because the \textit{de novo} pathway is
inefficient, but rather because it sets limits on the number of centrioles produced during each individual cell cycle.\textsuperscript{56}

We believe that Red-Br-nos displays several features that set it apart from conventional DNA-damaging drugs. Unlike doxorubicin, which causes CA during a chronic S-phase arrest, Red-Br-nos forces cells to transit through S phase and subsequently override the transient G2/M arrest to progress into mitosis despite DNA damage, although they eventually die via ‘metaphase catastrophe’. Thus, Red-Br-nos distinguishes itself from other traditional DNA-damaging agents, although it does act similar to UCN-01.\textsuperscript{34} Moreover, it has been shown that DNA-damage-induced cell death is enhanced by progression through mitosis,\textsuperscript{20} a conclusion, which is corroborated by our observations.

Intriguingly, a slight structural alteration (reduction of the lactone ring to a cyclic ether) of Red-Br-nos compared with noscapine and its congener, bromonoscapine, remarkably intensifies CA and drives SM and ‘metaphase catastrophe’. Presumably, the power of Red-Br-nos to amplify centrosomes and cause multipolarity translates into superior chemotherapeutic strength, as a recent NCI screen revealed that Red-Br-nos is significantly more potent than noscapine in virtually all cancer lines tested (data not shown). Nonetheless, Red-Br-nos retains the non-toxic attributes of the mildly declustering parent, noscapine, and spares normal cells.\textsuperscript{18} This is perhaps because normal cells, owing to robust checkpoints, have durable mechanisms that prevent cell-cycle progression in the presence of damaged DNA. In contrast, cancer cells have leaky checkpoints, which they invoke but fail to sustain, and thus march through the cell cycle with lethal consequences. Red-Br-nos-induced downmodulation of MT dynamicity in healthy cells may be harmless because normal checkpoint systems mitigate the impact. In contrast, cancer
cells may ‘ignore’ the impairment to their detriment. We speculate that the leakiness of cancer cell checkpoints combined with the mild impact of Red-Br-nos on MT dynamicity constitute the basis of its cancer cell selectivity and nontoxicity, respectively. We are optimistic that Red-Br-nos can serve as an invaluable tool to gain insights into de novo centriole formation and molecular mechanisms that normally restrict centriole numbers.

4.6 References


5. OVEREXPRESSION OF THE KINESIN-14 PROTEIN HSET FUELS TUMOR PROGRESSION VIA CENTROSOme CLUSTERING-INDEPENDENT MECHANISMS AND PREDICTS POOR CLINICAL OUTCOMES IN BREAST CANCER PATIENTS

5.1 Abstract

Amplified centrosomes are widely recognized as a hallmark of cancer and human breast tumors, in particular, harbor supernumerary centrosomes in almost 80% of tumor cells. Although amplified centrosomes potentially compromise cell viability by compelling cells to form multipolar spindles resulting in death-inducing aneuploidy, cancer cells cleverly turn things in their favor by clustering extra centrosomes during mitosis. As a result, cancer cells display bipolar spindle phenotypes and derive the collateral benefit of maintaining a tolerable level of aneuploidy, an edge to their survival. HSET/KifC1, a kinesin-like minus-end directed microtubule motor has recently found fame as a crucial centrosome clustering molecule. Here we show that HSET promotes tumor progression via multiple mechanisms, some of which are independent of centrosome clustering. We found that HSET is overexpressed in several human cancers including breast carcinomas wherein nuclear HSET accumulation correlated with histological grade. High nuclear HSET expression in patients with breast cancer predicted increased risk of disease progression, poor progression-free (PFS) and overall survival (OS). In addition, deregulated HSET protein expression in breast cancers was associated with gene amplification and/or translocation. Our data also provide compelling evidence that HSET overexpression is pro-proliferative, promotes clonogenic survival and enhances kinetics of progression through G2 and M-phases of the cell cycle. Importantly, HSET co-
immunoprecipitates with surviving, a pro-proliferative and pro-survival protein, and its overexpression protects survivin from proteasome-mediated degradation, leading to increased steady-state levels of survivin. Our observations provide the first evidence of centrosome-clustering independent activities of HSET that fuel tumor progression and firmly establish HSET overexpression as a strong driver of tumor evolution. Our findings thus underscore the significance of HSET both as a potential prognostic biomarker and as a valuable cancer-selective therapeutic target.

5.2 Introduction

About 80% of invasive breast cancers exhibit supernumerary centrosomes, a feature commonly referred to as centrosome amplification. Most breast cancers harbor abnormalities in centrosome structure, function, or localization within the cell. These abnormalities can potentially cause cytoarchitectural distortion in cancer tissues with loss of cellular differentiation (anaplasia) via altered centrosome function in microtubule nucleation and organization. Centrosome amplification can initiate tumorigenesis in Drosophila neuroblasts; thus, it is becoming recognized that centrosome amplification is one of the primary causes of breast cancer and is not just a consequence of malignant transformation.

The presence of more than two centrosomes within a cell can pose a grave conundrum as it may lead to the assembly of a multipolar mitotic spindle, and the production of nonviable progeny cells due to lethal levels of chromosomal loss (i.e., death-inducing, high-grade aneuploidy). However, cancer cells harboring extra centrosomes circumvent these catastrophic consequences and survive. The secret to their survival and success, as it turns out, lies in a clever tactic that cancer cells use to sidestep spindle multipolarity, viz., centrosome clustering, whereby
the excess centrosomes are artfully corralled into two polar foci to enable formation of a pseudo-
bipolar mitotic spindle\textsuperscript{5, 6}. During a preceding, transient, multipolar state, merotelic kinetochore-
microtubule attachments occur, thus engendering low-grade whole chromosome missegregation
that could be ‘tumor-promoting’\textsuperscript{7}.

HSET/KifC1, a minus end-directed motor protein that promotes microtubule cross-
linking, sliding, bundling and spindle pole focusing, has been recently identified as an essential
mediator of supernumerary centrosome clustering in cancer cells\textsuperscript{8}. HSET has also been shown to
be indispensable for the clustering of acentrosomal microtubule organizing centers (MTOCs)
whose production tends to be hyperactivated in cancer cells. HSET knockdown in cells with
supernumerary centrosomes causes the excess centrosomes to be scattered by pole-separating
forces, leading to rampant spindle multipolarity and cell death\textsuperscript{9}. By contrast, HSET function
appears to be non-essential in healthy somatic cells due to the presence of two centrosomes that
shoulder the responsibility of bipolar spindle assembly. In cells devoid of centrosomes, such as
oocytes, HSET function is indispensable for the assembly of a fusiform bipolar spindle\textsuperscript{10}.

The transcription of HSET is known to be under the regulation of the cell cycle
regulatory transcription factor, p110Cux1\textsuperscript{11}. HSET’s localization changes dynamically during
cell cycle progression; HSET is sequestered in the nucleus in interphase, presumably to avoid
untimely microtubule cross-linking\textsuperscript{12}. Upon nuclear envelope breakdown at the onset of mitosis,
HSET is released into the cytoplasm to resume its activities in bipolar spindle biogenesis. During
mitosis, HSET is localized both on the spindle poles and along the spindle length. With mitotic
spindle breakdown in telophase, HSET is localized on the minus-end of microtubules near the
spindle poles before being shuttled back into the nucleus\textsuperscript{12, 13}. HSET transport inside the nucleus
is regulated by Ran GTPase via association of the bipartite Nuclear Localization Signal of HSET
with nuclear import receptors importin α/β. HSET has been identified as an ubiquitination substrate of APC/C-Cdh1 complex in *Xenopus* extracts, suggesting its degradation during mitotic exit.

Recently, attention has converged on HSET as a potential chemotherapeutic target due to its intriguing association with malignancy. RT-PCR studies have shown that HSET’s expression level in lung cancer is associated with increased risk of metastatic dissemination to the brain. While docetaxel resistance in breast cancer is also suggested to be partly mediated by HSET. HSET expression is also higher in triple negative breast cancers compared to non-triple negative ones, suggesting its role in tumor aggressiveness. The differential dependence of cancer cells on HSET for viability and association of HSET expression with metastases, all raise the tantalizing possibility that HSET may play an important role in tumor progression than previously appreciated. However, more direct evidence of HSET’s role in clinical progression of breast cancer and mechanistic studies revealing the molecular circuitry involved therein are lacking.

In this study, we evaluated HSET expression in breast carcinomas and examined its association with clinical tumor progression. Intriguingly, we found that HSET overexpression at the time of diagnosis was significantly associated with worse prognosis and overall survival. Exploration of its mechanistic role in tumor progression unmasked plausible centrosome clustering-independent roles of HSET underlying enhanced tumor cell proliferation and survival, and disease progression. Our results substantiate the idea that HSET could be an invaluable, cancer-cell selective therapeutic target and may serve as a prognostic biomarker for breast cancer.
5.3 Materials and Methods

5.3.1 In silico analysis of HSET gene expression:

5.3.1.1 Data Collection: One channel microarray data for various cancers were collected from Gene Expression Omnibus (GEO) database. The list of the GSE ID’s is given in following table.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Normal Samples GEO Series ID</th>
<th>Cancer Samples GEO Series ID</th>
<th>N Normal</th>
<th>N Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
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<td>GSE10878</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td><a href="http://www.broadinstitute.org/mpr/publications/projects/Lung_Cancer/">http://www.broadinstitute.org/mpr/publications/projects/Lung_Cancer/</a></td>
<td>GSE10878</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>GSE10797</td>
<td>GSE7390, GSE18864</td>
<td>16</td>
<td>179</td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>GSE4107</td>
<td>GSE18088</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>Cervical Cancer</td>
<td>GSE9750</td>
<td>GSE9750</td>
<td>21</td>
<td>33</td>
</tr>
</tbody>
</table>

5.3.1.2 Data pre-processing: One channel microarray data was Robust Multiarray normalized (RMA), and was further taken for processing.

5.3.1.3 Analysis of HSET gene expression: Logarithm to the base 2 transformed HSET expression levels are plotted in Fig. 1 for each of the glioblastoma, lung, breast, colon, cervical cancers and leukemia patients compared to their normal pairs.

5.3.2 Clinical tissue samples:

All paraffin-embedded tissue slides were commercially obtained (from Accumax, and US Biomax). A subset of well-annotated tissue microarrays (193 biospecimens) with information on
clinical outcomes, were obtained from Gabriela Oprea, Grady Memorial Hospital. The Emory Institutional Review Board (IRB) approval was obtained for all aspects of the study.

5.3.3 Immunohistochemistry, Scoring and Statistics:

Corresponding to the identified breast cancer cases, formalin-fixed and paraffin-embedded tissues were used to construct Tissue microarrays (TMAs) containing 2 cores for each case (1 mm in diameter, 5 micron thick sections) with adjacent normal tissues. For immunohistochemical staining, the TMAs were first deparaffinized and then rehydrated in a series of ethanol baths (100%, 90%, 75% and 50%). Antigen retrieval was achieved by citrate buffer (pH 6.0) in a pressure-cooker (15 psi) for 3 min. Immunostaining for HSET (1:1000 dilution) was performed using a rabbit polyclonal antibody, which was a generous gift from Claire Walczak (Indiana University). Enzymatic antibody detection was performed using Universal LSAB + kit/HRP (DAKO, CA, USA). HSET staining was scored for both the nuclear and cytoplasmic localization as an intensity and frequency score by an experienced pathologist. A relative intensity score was represented as 0 = none, 1 = low, 2 = moderate, or 3 = high and frequency score was represented as the percentage of cell nuclei or cytoplasms demonstrating HSET positivity (i.e., a score of 1, 2, or 3). The product of the relative intensity and frequency was measured as HSET weighted index (WI) for both the nucleus and cytoplasm. HSET WI was then assessed with various clinical parameters (Nottingham Grade) and survival outcomes (OS and PFS).

5.3.4 Statistical Methods:

Statistical analyses were performed using SAS Version 9.3 with HSET WI considered as a continuous variable in all the required analysis tests. Kaplan-Meier survival curves were
generated for patient outcomes (OS and PFS) stratified by negative and positive HSET WI groups. Survival differences between the groups were assessed using the log-rank test.

5.3.5 Cell culture and transfection:

HeLa-HSET-GFP cells were generously provided by Claire Walczak (Indiana University). HeLa and HeLa-HSET-GFP, MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Briefly, cells were seeded onto 100-mm plates 1 day prior to transfection. Plasmid DNA (5 μg) and 15 μl of DharmaFECT 4 transfection reagent (Thermo Scientific, PA, USA) were used for each transfection. HSET-pEGFP plasmid was generously provided by Dr. Claire Walczak. Cells overexpressing HSET were selected in the medium containing G418 (400 μg/ml). The G418-resistant colonies were collected and examined for HSET expression. SMARTpool: ON-TARGETplus KIFC1 siRNA (Dharmacon, PA, USA) was used to knockdown HSET in MDA-MB-231 cells.

5.3.6 Cellular protein preparation, Western blotting, Immunofluorescence and antibodies:

Cells were cultured to ~70% confluence and protein lysates were collected following transfection or otherwise. Fresh frozen tissue sections were first sonicated and lysates were then prepared. The immune-reactive bands corresponding to respective primary antibodies were visualized by the Pierce ECL chemiluminescence detection kit (Thermo Scientific). β-actin was used as loading control. For immunofluorescence staining, cells grown on glass coverslips were fixed with cold (−20 °C) methanol for 10 min and blocked by incubating with 2% bovine serum albumin/PBS.0.05% Triton X-100 at 37 °C for 1h. Specific primary antibodies were incubated with coverslips for 1h at 37 °C at the recommended dilution. The cells were washed with 2% bovine serum albumin/PBS for 10 min at room temperature before incubating with a 1:2000 dilution of Alexa 488- or 555-conjugated secondary antibodies. Cells were mounted with
Prolong Gold antifade reagent that contains 4′,6-diamidino-2-phenylindole (Invitrogen). Polyclonal rabbit anti-HSET antibody was provided by Dr. Claire Walczak. Antibodies against α-tubulin and β-actin were from Sigma (St. Louis, MO, USA). Antibodies against γ-tubulin, α-tubulin and β-actin were from Sigma (St. Louis, MO, USA). Anti-Mad2 antibody was from BD Biosciences (Pharmingen, San Jose, CA, USA). Antibodies against p-Bcl2 and cleaved caspase-3 were from Cell Signaling (Danvers, MA, USA). Alexa 488- or 555- conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA). Anti-Mad1 antibody was a generous gift from Andrea Musacchio. Anti-Ki67 antibody was from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

5.3.7 Kinase activity assay:

To examine cdk1 kinase activity, anti-cdk1 antibody was used to selectively immunoprecipitate cdk1-containing complexes from HeLa and HeLa-HSET-GFP cell lysates. The resulting immunoprecipitate was incubated with pure histone-H3 protein in the presence of p32-labelled ATP and kinase buffer. The kinase assay reaction allowed immunoprecipitated cdk1 to phosphorylate histone-H3 in vitro, the extent of which was measured by immunoblotting using phosphohistone-H3 antibody from Cell Signaling (MA, USA). Histone-H3 protein was from Millipore (MA, USA) and ATP was from Cell Signaling.

5.3.8 Fluorescence in situ Hybridization:

The slide samples from tumor cell lines or tumor tissue were hybridized by 2-color FISH with an HSET-specific BAC probe (RPCI-11 602P21, green) and a chromosome 6 centromere (CH514-7B4, red) (BACPAC). The HSET and centromere 6 probes were labeled with Cy3-dUTP (red) and FITC-dUTP (green), respectively, and hybridized with nuclei from cell lines or
tumor tissue samples. Plasmids for production of a particular FISH probe were combined in equimolar amounts (55–70 pM). Nick translation was performed on 2 µg of this substrate by using Nick translation kit (Abbott Molecular, IL, USA). The translation product was denatured for 3 mins at 95°C followed by fast cooling on ice and confirmed in 1.5% agarose gel electrophoresis as a smear of fragments ranging between 100 and 300 bp. A 2 min denaturation at 76°C was followed by overnight (12–16h) incubation at 37°C. Hybridization of the FISH probes was carried out in LSI/WCP hybridization buffer (Abbott Molecular, IL, USA). The slides were counterstained with DAPI (Invitrogen, NY, USA) and Zeiss LSM 700 confocal microscope was used to capture FISH images. Results were expressed as a ratio of the number of copies of the HSET gene to the number of chromosome 6–centromeric markers.

5.3.9 Flow cytometry:

Trypsinized cells were resuspended in PBS at 10⁶ cells/ml. Cells were then fixed by addition of ice-cold 70% ethanol. Ethanol-fixed cells were kept overnight at 4°C before staining. Cells were pelleted and washed twice with PBS. Cell pellets were incubated for an hour at room temperature with mouse anti-MPM-2 antibody (Millipore, MA, USA), followed by 1h incubation with Alexa-488 anti-mouse secondary antibody (Life Technologies, NY, USA). Finally cells were washed, pelleted and resuspended in propidium iodide-containing isotonic buffer (0.1 mg/ml) and 0.5% Triton X-100. Cell cycle distribution was determined by flow cytometry using an LSRFortessa Flow cytometer (BD Biosciences, CA, USA) and analyzed using Flowjo software (Tree Star, OR, USA).

5.3.10 Trypan Blue cell exclusion assay:

Cells were cultured to ~70% confluence followed by centrifuging and pellet was resuspended in 1 ml culture medium. 0.1 mL of 0.4% trypan blue solution was then added to 1
mL of cell suspension. Hemacytometer was loaded with 10 µl of the solution and examined immediately under a microscope. Live (white) and dead (blue) cells were counted and % cell viability was calculated using the following formula: % viable cells = \[1.00 – (\text{Number of live cells} ÷ \text{Number of total cells})\] × 100.

5.3.11 BrdU incorporation assay:

Asynchronous proliferating HeLa and HeLa-HSET-GFP cells were grown on coverslips to a confluency of ~70% and then incorporated with 10µM BrdU for 1h followed by fixation with 70% ethanol at room temperature and immersion in 0.07 N NaOH for 2 minutes (which was then neutralized with PBS, pH 8.5). Coverslips were then incubated in 2% bovine serum albumin/PBS.0.05% Triton X-100 at 37 °C for 1h followed by immunostaining using a 1:1000 dilution of Anti-BrdU-FITC antibody (BD Biosciences, San Jose, CA, USA). BrdU positive cells, indicative of cell proliferation, were captured on a Zeiss Axioplan-2 fluorescence microscope (20X).

5.3.12 Immunoprecipitation and endogenous ubiquitination analysis:

MDA-MB-231 cells were transiently transfected with control vector, HSET-pEGFP plasmid or SMARTpool siRNA as described above and lysates were collected. Cell lysates were clarified by centrifugation at 10,000 rpm, and the supernatants (500 µg of protein) were subjected to immunoprecipitation with 4 µl of anti-HSET or anti-survivin antibodies. After overnight incubation at 4°C, protein A-agarose beads were added and left at 4°C overnight. Immunocomplexes were then subjected to Western blot analysis as described previously. Western blot analysis with anti-ubiquitin antibody (Life Sensors, PA, 1:500) was performed by first incubating the PVDF membrane with 0.5% glutaraldehyde/PBS pH 7.0 for 20 min and then probing for the antibody.
5.4 Results

5.4.1 HSET is overexpressed in variety of human cancers

Given the crucial requirement of centrosome clustering mechanisms for the viability of cancer cells with extra centrosomes, we first wanted to examine the abundance of the clustering protein HSET in various cancers that harbor extra centrosomes. We envision that cancer cells would derive multiple benefits by upregulating HSET expression, in that it would not only permit clustering of extra centrosomes but also facilitate maintenance of low-grade aneuploidy that fosters cell viability and allows malignant transformation and tumor evolution. This rationale led us to hypothesize that cancers may have upregulated HSET expression compared to normal tissues. We performed an in silico gene expression analysis using publically-available microarray data to determine the expression level of HSET in various cancer tissue types. One-channel microarray data for glioblastoma, leukemia, lung and breast cancer patients with their normal sample pairs were collected from Gene Expression Omnibus (GEO) database\(^\text{18}\). Each of these samples were then Robust Multiarray (RMA) normalized\(^\text{19}\), and their logarithm to base 2-transformed HSET gene expression values were plotted to determine the difference as shown in Fig. 5.4.1A(i-vi). Next, we determined differences in HSET gene expression for cancer and normal sample groups using two-tailed test of hypothesis. Our statistical results indicated higher HSET gene expression in glioblastoma, leukemia, lung, breast, colon and cervical tumor samples as compared to their corresponding normal tissues. All these tumor types have been shown in various studies to exhibit significant degrees of centrosome amplification\(^\text{20-29}\). The average HSET expression for glioblastoma (N=20) and colon cancer (N=53) patients was found to be \(~3\)-fold higher than normal samples (N=3 and 10, respectively) (p<0.005), followed by breast cancer
patients (N=179) with more than 5-fold higher expression in tumors than in normal samples (N=16) (p<0.001). Our in silico results were consistent with observations from a previous study wherein HSET mRNA expression was significantly elevated in a broad panel of primary tumor tissue compared to corresponding normal tissue\textsuperscript{9}. Our in silico data corroborates with immunohistochemical analysis suggesting a significantly higher HSET expression in glioblastoma, colon and cervical tumor (Fig. 5.4.1Bii, Cii, Dii) as compared to their respective adjacent normal tissue samples (Fig. 5.4.1Bi, Ci, Di). These data suggest HSET overexpression as a general feature of cancers exhibiting significant centrosome amplification.
Figure 5.4.1 HSET overexpression in human carcinomas.

(A) Scatter plots depicting HSET gene expression in normal (green dots) versus tumor (red dots) tissues in (i) Glioblastoma, (ii) Lung carcinoma, (iii) Leukemia, (iv) breast carcinoma, (v) colon carcinoma and (vi) cervical carcinoma. Data was obtained from one channel microarray available at GEO database. Robust multiarray normalization was performed to obtain the differences depicted in the plots. 

(B, C and D) Immunohistographs showing HSET expression in glioblastoma tissue where a representative normal tissue(N) (Bi) is compared to tumor tissue(T)
(Bii); in colon tumor (Cii) versus adjacent normal (Ci) tissue; and in cervical tumor (Dii) versus adjacent normal (Di) tissue. Red arrows indicate positive nuclear HSET staining.

5.4.2 HSET is overexpressed in human breast cancers

Our in silico analyses of microarray data showed that breast cancers display significantly higher HSET expression (~5-fold) than corresponding normal tissue. In addition, given the pronounced occurrence of amplified centrosomes and centrosome clustering in aggressive breast cancer, we decided to focus our study on breast cancers. To explore the role, if any, of HSET in tumor progression and to analyze if this role was wholly dependent on its known function clustering supernumerary centrosomes, we examined whether HSET was overexpressed in human breast tumors. We immunoblotted 16 fresh-frozen human tumor samples and their paired adjacent normal tissues for HSET. An enhanced expression of HSET was observed in 10 tumor samples compared to their normal adjacent tissues and 7 representative normal/tumor sample pairs are shown in Fig. 5.4.2A. The remaining 6 normal/tumor pairs showed negligible overexpression of HSET (data not shown). Additionally, HSET expression in most human breast cancer cell lines was much higher than in non-cancerous or pre-malignant cell lines such as NIH3T3 and MCF10 series (MCF10A, MCF10AT1, MCF10DCIS) (Fig. 5.4.2B), indicating that HSET overexpression typifies breast cancer cells.
Figure 5.4.2 The HSET gene is overexpressed in breast cancers.

(A) Cell lysates from 16 paired clinical breast tumor tissues (T) and normal adjacent tissues (N) were analyzed for HSET protein expression by western blotting. Representative results of 7 paired samples are shown. (B) Immunoblot showing HSET expression in a) MCF10A series of cell lines representing continuum from near-normal breast (MCF-10A) to pre-malignant (MCF10-AT1) to comedo ductal carcinoma *in situ* (MCF10-DCIS), aggressive breast cancer cell lines such as MDA-MD-231 and T47D and normal mouse fibroblast cell line, 3T3. (C) Representative confocal micrographs showing depicting fluorescence in situ hybridization of two bacterial artificial chromosome (BAC) probes, one from the HSET locus on chromosome 6 (RPCI-11 602P21, green) and one from the chromosome 6 centromere (CH514-7B4, red), to paraffin-embedded primary breast tumor tissues. Amplifications of HSET were visualized as an increase in the number of green signals (denoted as G) relative to the number of red control centromere signals (denoted as R), where 1R1G and 2R2G represent normal HSET gene copy numbers and 1R4G, 2R4G, 2R5G, 1R5G, etc. represent instances wherein HSET gene locus is amplified. (D) Bar graph representation of various combinations of red and green copy numbers observed for HSET locus and chromosome 6 centromere as determined by visual quantitation from confocal images. 1R1G and 2R2G are considered normal copy numbers; elevated copy numbers but same ratio of R and G signals are considered as aneuploid (3R3G, 4R4G) and all other combinations with higher G-to-R ratios are considered as representing instances where the HSET gene is amplified.

Since higher HSET protein levels could arise either from an upregulation of transcription from the endogenous locus and/or an amplification of the locus encoding HSET, we decided to examine the copy numbers of the locus encoding HSET gene in normal and breast tumor tissues. We performed fluorescence in situ hybridization (FISH) to directly evaluate HSET copy number per cell in paraffin-embedded breast tumor tissues. We hybridized two bacterial artificial chromosome (BAC) probes to primary breast tumor tissues, one from the HSET locus on
chromosome 6 (RPCI-11 602P21, green) and one from the chromosome 6 centromere (CH514-7B4, red). Amplification of HSET was visualized as an increase in the number of HSET signals relative to the number of control centromere signals. We scored HSET amplification by FISH in four breast tumor tissues and found that, among them, three tumors exhibited HSET amplifications. No amplification of the HSET locus was observed in the normal adjacent tissues in these samples. We observed various types of copy number changes associated with HSET as shown in Fig 5.4.2C,D. FISH with the centromere probe indicated that most increases in HSET loci were not due to polyploidy of chromosome 6; rather, only 5% of cells were aneuploid. 38% of cells (500 cells each were counted from 2 tissue samples) showed 3 or more copies of HSET paired with only 1 or 2 copies of the centromere (Fig. 5.4.2D). More so, cancer cells isolated from fresh human breast tumor also showed HSET amplification (data not shown). These findings indicated alterations in the HSET gene copy number during tumorigenesis. It is worth mentioning that HSET gene amplifications in specific breast tumor samples were correlated with increased expression of HSET protein in all those samples using immunoblotting methods (data not shown).

5.4.3 **HSET overexpression correlates with breast cancer progression and aggressiveness**

Next we asked how HSET overexpression correlates with breast cancer progression and cancer aggressiveness. Using an immunohistochemical approach, we stained a total of 60 clinical specimens with 10 cases each of normal breast, ductal hyperplasia (DH), atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS), invasive breast carcinoma (low-grade) and invasive breast carcinoma (high-grade). In consonance with our immunoblotting data, our immunohistochemical analysis showed that HSET is selectively overexpressed in human breast cancers with negligible or absence of expression in normal breast epithelia (Fig 5.4.3A).
Intriguingly, we observed a selective increase in nuclear staining of HSET in tumor samples. Among subtypes based on varying types and extent of intraductal proliferation, we found a progressive increase in HSET nuclear staining intensity and frequency from ductal hyperplasia (DH) (Fig. 5.4.3Aii) to atypical ductal hyperplasia (ADH) (Fig. 5.4.3Aiii) to ductal carcinoma in situ (DCIS) (Fig. 5.4.3Aiv). In invasive breast cancers (both low- and high- grade), HSET nuclear staining was remarkably intense, with a significant increase in the number of positively stained nuclei per field in high-grade cancers (Fig. 5.4.3Av,vi) compared to low-grade ones (Fig. 5.4.3Aii,iii,iv). Majority of normal breast tissue samples (85%) showed no staining for HSET, while the remainder showing very weak staining (Fig. 5.4.3Ai, data not shown). We then calculated a weighted index score (WI) for HSET expression as a product of staining intensity score (+1, +2, +3) and percentage positive nuclei for each sample. HSET WI serves as an independent measure of the strength of HSET protein expression across all breast tumor specimens. Nuclear HSET WI values were then correlated with normal and tumor samples and also with the grade of tumor samples. Interestingly, nuclear HSET WI showed a strong correlation with increasing tumor grade in breast cancer (Fig. 5.4.3Bi,ii). Collectively, these observations indicate robust HSET overexpression in human breast tumors suggesting that abnormal HSET levels correlate with breast cancer development and HSET might play a role in progression of tumors into more malignant and aggressive forms. Having established a significant correlation between HSET expression and tumor differentiation, we next asked if there was an association of nuclear HSET WI with progression-free survival (PFS) and overall survival (OS) in breast cancer patients. While PFS was calculated as the number of days from diagnosis to the first local recurrence or metastasis if one occurred, or the last follow-up if the patient did not progress, OS was based on the number of days from diagnosis to death or last
follow-up if death was not recorded. Nuclear HSET WI was also categorized into high and low groups based on the median. Irrespective of the receptor status (n = 163), those with higher nuclear HSET WI (shown as HSET WI positive in Fig. 5.4.3Ci,ii) had statistically shorter PFS (p= 0.0034) and OS (p=0.0412) than patients with lower nuclear HSET WI (shown as HSET WI negative in Fig) (Fig. 5.4.3Ci,ii), clearly demonstrating that higher nuclear HSET expression levels significantly correlate with poorer clinical outcomes.
Figure 5.4.3 HSET overexpression correlates to increased aggressiveness and poorer clinical outcomes. Immunohistographs showing HSET expression in (Ai) normal breast, (Aii) ductal hyperplasia, (Aiii) atypical ductal hyperplasia, (Aiv) ductal carcinoma in-situ, (Av) invasive ductal carcinoma, low-grade, (Avi) invasive ductal carcinoma, high-grade. Brown (DAB) color shows HSET staining. Intensities of nuclear HSET staining were quantified using image analysis Aperio Image Scope v.6.25 software. A weighted index score (WI) for HSET expression was calculated and was assessed in 384 breast cancer and 19 normal samples. Box-whisker plots showing (Bi) HSET WI score in normal breast and tumor samples, (Bii) HSET WI score across
Grade I (n=40), Grade II (n=237) and Grade III (n=62) breast cancer samples. (Ci) Probability of progression free survival of 163 breast cancer patients with HSET nuclear expression above or below the median HSET weighted index (WI) value referred to as positive and negative, respectively (p=0.0034). (Cii) Probability of overall survival of 163 patients with positive and negative HSET weighted index (p=0.0412). Statistical analysis was conducted using SAS Version 9.3. Scale bar=10 µm. Red arrows indicate positive nuclear HSET staining.

5.4.4 HSET overexpression is associated with enhanced cell proliferation

Since elevated HSET expression exhibits a strong correlation with clinical development and progression of cancer, we next asked if high HSET levels had any impact on the kinetics of cancer cell proliferation in vitro. To this end, we used HeLa cells stably transfected with HSET-GFP to examine and compare the levels of various cell proliferation markers in HeLa-HSET-GFP and HeLa cells. Using immunoblotting methods, we found that levels of Ki67 (found in G1, S, G2 and M-phases of the cell cycle but is absent in G0 cells) was substantially elevated in HeLa-HSET-GFP cells compared to wild-type HeLa cells (Fig. 5.4.4A). This finding was consistent with the strikingly higher Ki67 labeling index observed in HeLa-HSET-GFP cells via immunostaining (Fig. 5.4.4B). This observation is noteworthy since the Ki67 labeling index often correlates with the clinical course of cancer, owing to which the proportion of Ki67-positive cells in a cell population has strong prognostic value for survival and tumor recurrence in cancer patients. Immunofluorescent staining for BrdU, a marker for cells undergoing S phase, also showed that a greater proportion of HeLa-HSET-GFP cells were BrdU-positive compared to wild-type HeLa cells (Fig. 5.4.4C). A visual quantitation of these observations revealed significantly elevated levels of Ki67 expression and BrdU incorporation in HeLa-HSET-GFP cells as compared to HeLa cells (Fig. 5.4.4D). We also observed enhanced Cdk1 activity and higher expression of phosphorylated histone-H3 in HeLa-HSET-GFP cells compared to HeLa cells, which is indicative of a larger proportion of cells in the HeLa-HSET-GFP line undergoing M-phase (Fig. 5.4.4A). All these lines of evidence strongly advocate for a pro-
proliferative role for HSET overexpression in the cellular context of cancer cells. HeLa-HSET-GFP cells also displayed significantly enhanced cell proliferation capacities when compared to wild-type HeLa cells in trypan blue assay. Equal numbers of each cell type were seeded on day 0 and were allowed to grow for 2 days (48h), and the number of cells were counted using trypan blue. Based on the data, the doubling time of HeLa-HSET-GFP cells was found to be ~11h as compared to ~16h for HeLa cells (Fig. 5.4.4E). We also performed colony formation assay with HeLa cells transiently transfected with control vector, HSET-GFP plasmid and HSET-GFP siRNA. HSET OE cells were able to form significantly higher number of colonies as compared to cells transfected with control vector. Much fewer colonies were observed upon HSET knockdown (KD). Similar proliferation effects were confirmed by colony formation assay in another breast cancer cell line, MDA-MB-231 upon transient HSET OE and KD (data not shown). Taken together, these data demonstrate that cells overexpressing HSET display enhanced cell proliferation, and suggest that HSET overexpression may confer significant proliferative advantages to cancer cells.
Figure 5.4.4 Cell proliferation is enhanced in HeLa cells that stably overexpress HSET.

(A) Immunoblots showing higher Ki67 and p-Histone H3 in HeLa-HSET-GFP (denoted as HeLa HSET) cells as compared to HeLa cells. Kinase activity assay showed higher cdk1 activity in HeLa-HSET-GFP cells as represented by the immunoblot showing enhanced phosphorylation of Histone H3 by cdk1 as compared to HeLa cells. The two bands representing HSET expression correspond to the endogenous HSET levels (lower band) and the GFP-HSET levels (upper band).

(B) Representative confocal immunomicrographs showing higher Ki-67 expression (red) in HeLa-HSET-GFP cells as compared to HeLa cells. (C) Randomly dividing HeLa-HSET-GFP and HeLa cells were incorporated with BrdU and immunostained with anti-BrdU antibody (green) to visualize the cells traversing S-phase. Representative immunofluorescence images showing higher BrdU incorporation in HeLa-HSET-GFP cells. (D) Bar graphs depicting the percent cells that are Ki-67 or BrdU positive in HeLa and HeLa-HSET cells. (E) Bar graphs representing number of cells in cell proliferation assay counted by trypan blue at Day 0 and Day 2 of seeding.

5.4.5 HSET overexpression leads to accelerated cell cycle kinetics

Since HSET OE enhances cellular proliferation in HeLa cells, we were curious to examine any changes in the cell cycle kinetics of cells that stably overexpress HSET (HeLa-HSET-GFP cells) compared to the parental ones. To this end, we synchronized HeLa and HeLa-HSET-GFP cells using a single thymidine block (19h) followed by 2-color flow cytometric
analysis of cell cycle profiles of HeLa-HSET-GFP and HeLa cells upon their release from the block at G1/S border. DNA content was analyzed with propidium iodide (PI) staining, where G2/M population was represented by double the intensity of PI (4N) compared to G1 cell population (2N). Anti-MPM-2 antibody tagged with Alexa-488 secondary antibody was used to detect a mitosis-specific marker (MPM-2), in order to distinguish between 4N DNA-bearing G2 and M populations. A close interval cell cycle profiling revealed that HeLa-HSET-GFP cells demonstrated faster cell cycle progression kinetics; in other words, the duration of one complete cell cycle was reduced in HSET-transfected cells (10.5h) as compared to wild-type cells (13h), with a stark shortening of the G2 and M phases (Fig. 5.4.5A,B,C). This trend was reflected when cyclin B1 levels (indicating mitotic phase) were followed in synchronized HeLa and HeLa-HSET-GFP cells using western blotting. While cyclin B1 levels surged at 10h followed by a decline in HeLa cells, they peaked at 8h and then declined in HeLa-HSET-GFP cells (Fig. 5.4.5D). Transient knockdown (KD) of HSET in HeLa cells resulted in a marginal decrease in cell cycle duration (14h as compared to 13h in HeLa cells) with a protracted G2/M phase (data not shown). This observation is in accordance with previously observed effects of HSET depletion in human fibroblast cells leading to delayed cyclin A degradation32.
Figure 5.4.5 HSET overexpression accelerates cell cycle kinetics.

(A) Cell cycle histograms representing cell cycle profiles of synchronized (i) HeLa and (ii) HeLa-HSET-GFP cells from the point of thymidine block release (0h) to the point after mitotic exit (14h and 11h, respectively). (B) Dot plots of PI (DNA) vs FITC (MPM-2) showing cells in G2 (lower box) and M-phase (upper box) specifically during the time of mitotic exit in (i) HeLa and (ii) HeLa-HSET-GFP cells. Two-color scatter plot (PI vs. GFP) shows two box gates, where the lower box represents G2 population (PI-4N and FITC negative) and upper box represents M population (PI-4N and FITC positive). G2/M population was represented by double the intensity of PI (4N) as compared to G1 population (2N). Mouse anti-MPM-2 antibody tagged with antimouse Alexa-488 secondary antibody was used as a mitosis-specific marker, to distinguish G2 and M population. The time for mitotic exit was determined by assessing the population in upper
gate of the 2-color scatter plot. A sudden surge in the proportion of mitotic population followed by a rapid fall indicated the time of mitotic exit. 13h was observed as the time of mitotic exit for HeLa cells whereas, 10.5h was the time of mitotic exit for HeLa-HSET-GFP cells. (C) Schematic depicting duration of each cell cycle phase in HeLa and HeLa-HSET-GFP cells assessed by flow cytometry following synchronization at G1/S border by single thymidine block. (D) Immunoblots showing cyclin B1 protein levels in synchronized HeLa and HeLa-HSET-GFP cells following release from thymidine block at G1/S boundary.

Most often, G1 phase contributes significantly to the cell cycle duration; thus we sought to determine the effect of HSET OE and KD on G1 phase kinetics. Upon gradual decrease of serum concentration from 10% to 0% over 24h and an additional 12hr serum starvation, transiently transfected HeLa control vector (CV), HeLa HSET OE and HeLa HSET KD cells were replenished with serum-containing medium and stained with “Cell-Clock” dye (Biocolor; a redox dye that changes color corresponding to distinct phases in cell cycle). Yellow cells in the culture represent G1 and their color changes to light green in S-phase. We followed the proportion of G1 (yellow-colored) cells from 0h (50-70% G1enrichment) to 9h after serum replenishment in all the three cases (CV, OE and KD). We observed negligible difference in the proportion of G1 cells in all three conditions (data not shown). This suggests that unlike G2 and M phase kinetics, HSET OE does not significantly affect the duration of G1 phase.

Faster kinetic progression of HeLa-HSET-GFP cells (through G2- and M) compared to HeLa cells raised the possibility that G2/M or spindle assembly checkpoint (SAC) functions may be compromised in HeLa-HSET-GFP cells. Mad1 is a critical component of the SAC along with Mad2, and an imbalance in the Mad1-Mad2 protein ratio results in a damaged SAC causing premature anaphase entry and chromosome instability. Interestingly, we found that HeLa-HSET-GFP cells express markedly higher levels of Mad1 with a distinct nuclear envelope localization compared to parental HeLa cells (Fig. 5.4.6A, B). This observation along with the known association of HSET with importins, indicate that HSET might be involved in regulating
mitotic entry and export\textsuperscript{35}. By contrast, there was no significant difference in the levels of Mad2 between the two cell lines (Fig. 5.4.6A), showing that the Mad1-Mad2 balance is highly perturbed in the HeLa-HSET-GFP cells. Thus we envision that excess HSET directly or indirectly incapacitates the SAC by disrupting the Mad1/Mad2 balance. The HeLa-HSET-GFP cells thus progress through the cell cycle rapidly in the presence of compromised checkpoints, which precipitates a greater likelihood of generating aneuploidy and thus, may accelerate the process of tumor evolution.

Our data from the HeLa-HSET-GFP cells demonstrated that HSET OE can markedly accelerate the kinetics of G2 and M phases (Fig. 5.4.5A,B,C). Intriguingly, our immunohistochemical data from clinical tumor samples (Fig. 5.4.3A), showed strong nuclear localization of HSET. In order to obtain a deeper understanding of how elevated HSET levels may hasten progression through G2 and M phases of the cell cycle and to exclude the possibility that faster kinetic progression through the cell cycle may result from artifactual mislocalization of HSET, we decided to examine in detail the sub-cellular localization of this intriguing protein in HeLa cells at various cell cycle stages. We found that HSET is conspicuously confined to the nucleus throughout interphase (data not shown). Our observations are consistent with the finding that the \textit{Xenopus} homolog of HSET, XCTK2, is sequestered in the nucleus in interphase in a Ran-dependent manner via the association of the NLS of XCTK2 with importin $\alpha/\beta$\textsuperscript{36}. In summary, the nuclear localization of the human HSET protein in interphase strongly suggest that the acceleration of the kinetics of G2 may be ascribed to a hitherto unknown activity of HSET within the nucleus.
5.4.6 HSET overexpression upregulates survival signaling in cancer cells

Tumor cell numbers and tumor growth are functions not only of cell proliferation rates but are also crucially influenced by cell survival and/or apoptosis. Having ascertained that HSET OE can enhance the kinetics of cell proliferation in tumors, we wanted to investigate whether elevated levels of HSET have any impact on the status of pro-survival signaling in HeLa cells. Immunoblots showed enhanced survival signaling as evidenced by notably high survivin and p-Bcl2 levels in HeLa-HSET-GFP cells (Fig. 5.4.6C) compared to levels seen in parental HeLa cells. To investigate if HSET OE affects signaling pathways that impinge on cell proliferation, adaptation to hypoxic environments, or cell survival in breast cancer cells, we compared levels of some key proliferation, hypoxia and cell survival markers in parental MDA-MB-231 cells with MDA-MB-231-HSET overexpressing cells and MDA-MB-231-HSET knockdown cells. We observed significantly enhanced levels of survivin and phospho-survivin, the hypoxia-induced factor HIF1α, the SAC protein Mad1 and the mitotic kinase Aurora-B in MDA-MB-231-HSET overexpressing cells (Fig. 5.4.6D). However, upon HSET knockdown, marginal or no reduction was observed in the expression levels of these proteins as compared to their respective levels in control cells (Fig. 5.4.6D). The differential effects observed upon HSET OE and KD indicated that HSET may not normally be a key regulator of proliferation and survival pathways. Several studies have in fact shown that HSET function is dispensable for the viability of non-cancerous cells. However, our OE data strongly suggest that an elevated level of HSET expression thrusts proliferation and survival signaling in cancer cells into an “overdrive” mode. In sum, while HSET plays a non-essential role in regulating survival signaling in cancer cells, HSET overexpression enhances both proliferation as well as survival of cancer cells and perhaps fuels tumor progression by providing cancer cells with a proliferation and survival advantage. Our
data thus provide evidence that cancer cells may employ auxiliary pathways/mechanisms, such as those involving the kinesin motor HSET, to their advantage.

Figure 5.4.6 HSET overexpression upregulates survival proteins and disrupts balance of checkpoint proteins. (A) Immunoblots showing HSET, Mad1 and Mad2 protein levels in HeLa and HeLa-HSET-GFP cells. β-actin is used as a loading control for all western blots. (B) Immunofluorescence micrographs showing Mad1 (green) levels and localization in HeLa and HeLa-HSET-GFP cells. (C) Immunoblots showing the expression levels of survival proteins (survivin, p-Bcl2) in HeLa
and HeLa-HSET-GFP cells. (D) Immunoblots showing the expression of proteins associated with cell survival, cell cycle regulation, spindle assembly checkpoint and adaptation to hypoxia in MDA-MB-231 cells (C) compared to MDA-MB-231 cells transiently transfected with HSET-pEGFP plasmid (OE). (E) Immunoblots showing HSET and cleaved caspase-3 protein expression in MDA-MB-231 cells transiently transfected with vehicle control, HSET pEGFP plasmid or HSET siRNA, followed by UV-C exposure at 25 J/m² for 10 min. (F) Immunoblots showing HSET and survivin protein levels in MDA-MB-231 with control vector (CV), with HSET overexpression and with HSET knockdown when HSET was immunoprecipitated (HSET IP) or not immunoprecipitated (beads only) followed by immunoblotting against survivin. (Fii) Immunoblots showing survivin immunoprecipitated from MDA-MB-231 cells (CV, OE and KD) and immunoblotted against surviving and ubiquitin. (G) Schematic model depicting the involvement of HSET in tumor progression and metastasis via a) previously established mitotic pathways (Green boxes) and interphase-specific pathways suggested by our data (blue boxes). Dotted arrow indicates an unknown and indirect modulation of various downstream pathways by overexpressed nuclear HSET. C= control GFP vector.

To further explore the physiological role of HSET in cell survival signaling, we assessed the ability of MDA-MB-231 cells with HSET OE or KD to resist UV-induced apoptosis. To this end, we transiently transfected MDA-MB-231 cells with control vector, HSET OE construct or HSET siRNA (~70% transfection efficiency) 24h prior to UV irradiation. Following 10 min exposure to UV-C at 25 J/m², cells were placed in the incubator for apoptosis induction for 5h. Lysates were then collected for determining HSET and cleaved caspase-3 protein levels (an early marker for apoptosis induction) and cell viability was determined using trypan blue assay. Western blot analysis revealed significantly higher cleaved caspase-3 induction in cells with HSET knock-down, whereas cells with HSET OE showed slightly lower cleaved caspase-3 levels as compared to cells transfected with control vector (Fig. 5.4.6E). These data indicate the ability of HSET overexpression to promote cell survival in cancer cells.

5.4.7 HSET overexpression increases steady-state levels of survivin by decreasing the latter’s poly-ubiquitination

Since we observed extensive upregulation of survivin protein expression as a result of HSET OE and significant reduction upon HSET KD, we wanted to determine if HSET occurs in
the same protein complex as survivin and whether HSET OE has any effect on the APC/C-dependent proteolysis of survivin.

First, we tested if HSET and survivin co-immunoprecipitate with each other. We immunoprecipitated HSET from whole cell lysates of MDA-MB-231 cells that carried (i) a control vector, (ii) an HSET OE plasmid, and (iii) an HSET siRNA-bearing construct. Upon probing the immunoprecipitates for survivin, we confirmed that the anti-HSET antibody was able to pull down survivin in all the three cases, with an increased survivin pull down in the cell lysates overexpressing HSET (Fig. 5.4.6F). We also confirmed this association by immunoprecipitating survivin and in turn probing with HSET antibody (data not shown). These data indicate that HSET binds to survivin either directly or indirectly.

Since survivin’s role in prosurvival signaling is strongly regulated by its degradation via ubiquitination\textsuperscript{38}, we further set out to test the possibility that increased HSET binding to survivin protects survivin from ubiquitination and APC/C-dependent degradation. In MDA-MB-231 cells transiently transfected with control vector, HSET-GFP plasmid and HSET siRNA, we immunoprecipitated survivin and immunoblotted against survivin and ubiquitin. Intriguingly, we observed reduced polyubiquitin signals in HSET overexpressing cells, even though survivin protein levels were extensively overexpressed in HSET overexpressing cells (Fig. 5.4.6F) as observed earlier (Fig. 5.4.6D). We also observed marginally higher ubiquitin levels in HSET KD cells as compared to control, even though the survivin protein levels were comparable in both the cases. These observations, in sum, uncover a previously unrecognized role of HSET overexpression in tumor progression via supplementing prosurvival pathways.
5.5 Discussion

In recent years, the key role played by the kinesin-14 protein HSET/KifC1 in centrosome clustering in cancer cells with supernumerary centrosomes, has been well established. In addition to its mitotic spindle-specific roles, several other roles of HSET requiring or independent of its motor activity have been suggested. Minus-end directed motor activity of HSET has been implicated in the microtubule-based motility and processing of early endocytic vesicles\textsuperscript{39}. HSET also plays a crucial role in rat spermatogenesis in concert with nucleoporins and importin β\textsuperscript{40}. Recent studies have also suggested a role of HSET in intracellular active transport of bare double-stranded DNA\textsuperscript{41}. Although it is presently unclear whether HSET performs all these functions in cancer cells, these studies bring to light the possibility that HSET’s involvement in tumor biology could be multifaceted.

We (Fig. 5.4.1A,B) and others\textsuperscript{9} have found that a variety of primary tumors overexpress HSET as compared to their normal adjacent tissues. Several other threads of largely correlative and circumstantial evidence have suggested an involvement of HSET in driving tumor progression and metastases.\textsuperscript{9, 15} However, our study is the first to explore and obtain several new mechanistic insights into the pathology of excess HSET in breast cancer cells. We have firmly cemented the hitherto anecdotal evidence with experimental data to show that HSET OE in breast cancer (i) correlates strongly with aggressiveness of the disease, (ii) is attributable, at least in part, to amplification of the genomic locus for this gene, (iii) promotes tumor cell proliferation by accelerating cell cycle kinetics, and (iv) promotes pro-survival signaling and adaptation to hypoxic environments. Given the myriad clinical implications of these important findings, our study spotlights the tremendous potential that HSET presents both as a biomarker of tumor progression and as an invaluable cancer cell-specific therapeutic target.
Four critical observations lead us to believe that HSET might have additional roles in driving tumor progression, independent of its centrosome clustering/spindle pole focusing role in mitosis, viz.,  (i) elevated ‘nuclear’ expression of HSET predominantly in the interphase cells within high grade tumors as revealed by immunohistochemical staining suggests that HSET may perform critical mitosis-independent functions in aggressive tumors or plausibly lead to more aggressive phenotypes within tumors; (ii) overexpression of HSET results in accelerated G2 and M phases. Faster mitoses can conceivably arise from a severely compromised SAC function that presumably allows HSET-overexpressing cells to rapidly traverse mitosis in the presence of aberrations including chromosome attachment errors. However, we are aware of the caveat that this mitotic role of HSET does not provide an alibi for the observed faster progression through the G2-phase upon HSET overexpression; (iii) HSET OE in HeLa cells leads to faster cell kinetics and enhanced overall proliferation (Fig. 5.4.5A,B,C), and (iv) HSET OE leads to the upregulation of the expression of phospho-survivin, Bcl-2, HIF1α, Aurora B and Mad1, and presumably upregulates the signaling pathways that lie downstream of these key regulatory factors. Furthermore, since fewer than 3 percent of HeLa cells possess amplified centrosomes (our unpublished observations), we believe that the pro-proliferative role of HSET that we have demonstrated in our study in HeLa cells provides strong evidence for a centrosome clustering-independent activity of HSET.

To further support the centrosome-clustering independent aspect of HSET’s role in driving tumor survival and proliferation, we assessed the effects of HSET OE and KD in HeLa cells with or without centrosome amplification. We induced extra centrosomes in HeLa cells by aphidicolin treatment (20 µM for 48h) and then compared the effect of HSET OE on expression of proliferation/survival markers in the HeLa cell lines bearing normonumerary and
supernumerary centrosomes. The fact that we were able to show higher expression of proliferation and survival proteins upon HSET OE in the same cell line regardless of its centrosome status (data not shown), asserts the centrosome clustering-independent role of HSET in driving cell proliferation and survival. In sum, the afore-stated observations strongly support a nuclear and centrosome clustering-independent role of HSET in driving cell cycle kinetics and tumor proliferation in breast cancer cells.

Our data showed that HSET OE leads to an increase in Mad1 levels without any significant change in the levels of its partner protein, Mad2. We postulate that this surge in Mad1 protein levels (Fig. 5.4.6A,B) facilitates premature anaphase entry by titrating the soluble pool of Mad2 and thereby damaging SAC function, and provides a possible explanation for the speedier execution of mitosis in HeLa-HSET-GFP cells. Mad1 overexpression in HeLa cells has been shown to disrupt the stoichiometric balance between Mad1 and its partner Mad2 to severely cripple SAC function leading to aneuploidy and chromosomal instability, a condition from which cells with an advantage for proliferation tend to be selected. We therefore believe that HSET OE fuels aneuploidy and tumor evolution through both centrosome clustering-dependent and independent means, which are depicted in our schematic model (Fig. 5.4.6B).

In addition, our study has yielded several novel mechanistic insights regarding the signaling pathways governed by HSET. Our data indicate that HSET is not merely a kinesin motor with a centrosome clustering function, but is actually a key member of an oncprotein axis that includes HIF1α and Aurora B, and controls survival signaling through phospho-survivin and Bcl-2 (Fig. 5.4.6A, C, D). The dysregulation of HIF1α and Aurora B are implicated in many aspects of cancer development and advancement. HIF1α activates transcription of a slew of genes involved in crucial aspects of cancer biology, including angiogenesis, cell survival and
invasion and thus controls various aspects of tumor progression and responses to variations in microenvironmental oxygenation\textsuperscript{43}. Notably, HIF1α drives the expression of survivin which performs a dual function- it is an anti-apoptotic protein that additionally promotes cell proliferation\textsuperscript{44-46}. HSET OE is also associated with an upregulation of Aurora B which is a chromosomal passenger protein involved in chromosome segregation, spindle checkpoint, and cytokinesis\textsuperscript{47}. Aurora B overexpression, observed in several tumor types\textsuperscript{48}, has been linked with aggressive metastasis and poor prognosis of cancer patients\textsuperscript{49, 50}. Our data thus suggest that HSET OE-driven elevation in HIF1α and Aurora B kinase levels incites upregulation of the pro-proliferative and pro-survival signaling networks controlled by HIF1α and Aurora B, and together with the increased aneuploidy triggered by impaired SAC function, facilitates tumor evolution into more malignant forms.

Importantly, our immunoprecipitation experiments demonstrate that both HSET and survivin exist within the same complex in MDA-MB-231 cells. We further investigated the molecular and functional significance of HSET’s association with survivin and uncovered that HSET binding to survivin protects survivin from degradation by interfering with the latter’s ubiquitination. It has been shown that survivin ubiquitination and degradation occurs in the nucleus (Connell et al., JBC, 2008). We propose that high levels of nuclear HSET inhibit the ubiquitination-dependent proteolysis of survivin in the interphase nucleus of cancer cells. Survivin accumulation is known to increases Aurora B kinase activity which in turn, increases the endogenous levels of phosphorylated histone H3; clearly, we observe all these effects following HSET OE (Fig. 5.4.6). Thus, we have provided mechanistic evidence that HSET OE, by stabilizing survivin, leads concurrently to both increased cell proliferation and survival signaling.
A recent study revealed HSET as a transcriptional target of p110CUX1. CUX1 was also reported to regulate the expression of cell cycle-regulated genes such as those encoding p21WAF1, histones H1, H2A, H2B, H3, and H4, and DNA polymerase(Pol) α. Constitutive activation of p110CUX1 is known to drive cell proliferation by expediting entry into S phase. p110CUX1 has been known to interact with E2F1 transcription factor resulting in tight regulation of cell proliferation by transcriptional activation of several cell cycle regulatory genes. Interestingly, Mad1 is also shown to be transcriptionally regulated by p110CUX1. In light of these insights, we are unable to rule out the possibility that HSET nuclear overexpression and upregulation of Mad1 levels are a mere consequence of an upstream regulation by classical tumor promoting genes. The Cux1-E2F-HSET cell proliferation axis thus demands further exploration in order to substantiate the validity of this prospect. Besides, we cannot discount the significance of substantial cell cycle effects observed upon HSET overexpression and the unyielding relationship between HSET nuclear expression and patient survival.

Taken together, our results provide compelling evidence that HSET OE drives tumor progression through multiple mechanisms that include (i) enhancement of tumor cell proliferation rates, (ii) increasing aneuploidy through centrosome clustering, upregulation of Aurora B and compromised SAC function, (iii) promoting pro-survival signaling and adaptation to hypoxic conditions. Clearly, these findings that argue for the existence of a causative link between nuclear HSET accrual and tumor aggressiveness, have far-reaching clinical implications including unlocking the potential of HSET nuclear expression serving as a prognostic biomarker, and HSET taking shape as a cancer-selective therapeutic target for the design and preclinical development of small-molecule HSET inhibitors for non-toxic breast cancer therapy.
5.6 References


6. CENTROSONE DECLUSTERING DRUGS MEDIATE A TWO-PRONGED ATTACK ON INTERPHASE AND MITOSIS IN SUPERCENTROSONAL CANCER CELLS

6.1 Abstract

Classical anti-mitotic drugs have failed to translate their preclinical efficacy into clinical response in human trials. Their clinical failure has challenged the notion that tumor cells divide frequently at rates comparable to those of cancer cells in vitro and in xenograft models. Given the preponderance of interphase cells in clinical tumors, we asked whether targeting amplified centrosomes, which cancer cells carefully preserve in a tightly clustered conformation throughout interphase, presents a superior chemotherapeutic strategy that sabotages interphase-specific cellular activities such as migration. Herein we have utilized supercentrosomal N1E-115 murine neuroblastoma cells as a test-bed to study interphase centrosome declustering induced by putative declustering agents such as Reduced-9-bromonoscapine (RedBr-Nos), Griseofulvin and PJ-34. We found tight “supercentrosomal” clusters in interphase and mitosis of ~80% of patients’ tumor cells with excess centrosomes. RedBr-Nos was the strongest declustering agent with a declustering index (DI) of 0.36, and completely dispersed interphase centrosome clusters in N1E-115 cells. Interphase centrosome declustering caused inhibition of neurite formation, impairment of cell polarization and Golgi organization, disrupted cellular protrusions and focal adhesion contacts - factors that are crucial pre-requisites for directional migration. Thus our data illustrate an interphase-specific potential anti-migratory role of

2Parts of this chapter have been published verbatim in Cell Death and Disease. 2014 Nov 20;5:e1538. as “Centrosome declustering drugs mediate a two-pronged attack on interphase and mitosis in supercentrosomal cancer cells”.
centrosome declustering agents in addition to their previously acknowledged ability to induce spindle multipolarity and mitotic catastrophe. Centrosome declustering agents counter centrosome clustering to inhibit directional cell migration in interphase cells and set up multipolar mitotic catastrophe, suggesting that disbanding the nuclear-centrosome-Golgi axis is a potential anti-metastasis strategy.

6.2 Introduction

Unlike in vitro cell cultures, cancer cells in patients’ tumor tissues have low mitotic indices and proliferation rates\(^1\). Consequently, drugs targeting mitosis demonstrate limited clinical efficacy, which exposes a fundamental weakness in the rationale underlying their clinical development. By contrast, classical microtubule targeting agents (MTAs), largely believed to act by perturbing mitosis, remain the mainstay of chemotherapy in the clinic. Given the miniscule population of mitotic cells in patient tumors\(^2\)\(^-\)\(^3\), it stands to reason that MTAs must target interphase\(^4\). This paradigm shift has spurred an intense search for novel interphase targets that combine the “ideal” attributes of cancer-cell selectivity and the ability to confer vulnerability on a large proportion of tumor cells.

Centrosomes, the major microtubule organizing centers (MTOCs) of cells, are required for accurate cell division, cell motility, and cilia formation\(^5\). The number of centrosomes within a cell is strictly controlled, and their duplication occurs only once per cell cycle. Nearly all types of cancer cells have abnormal numbers of centrosomes\(^6\)\(^-\)\(^8\), which correlates with chromosomal instability during tumorigenesis\(^9\)\(^-\)\(^11\). Supernumerary centrosomes in cancer cells can cause spindle multipolarity and thus non-viable progeny. Cancer cells avoid this outcome by clustering centrosomes to assemble a pseudo-bipolar mitotic spindle, which yields viable daughter cells\(^12\).
Thus, disrupting centrosome clustering may selectively drive cancer cells with amplified centrosomes to mitotic catastrophe and apoptosis without affecting normal cells.

The fate and interphase role of the supercentrosomal cluster inherited by each daughter cell at the end of a pseudobipolar mitosis is unknown. This is an important research question because a majority of cells within tumors are in interphase and the centrosomes’ command over microtubule nucleation is crucial for cellular organization and motility in interphase. If cancer cells cluster centrosomes in interphase, then disrupting the cluster could impact interphase-specific processes, opening up a vital therapeutic avenue. We envision that centrosome declustering would a) derail interphase-specific polarization and migration processes and b) precipitate multipolar mitosis culminating in apoptosis. This two-pronged strategy would impact a significantly larger proportion of tumor cells and consign them to death. Our study herein establishes that centrosome declustering drugs (RedBr-Nos, Griseofulvin, and PJ-34) achieve this two-pronged attack as a unique class of agents that exhibit multiple cellular activities.

6.3 Materials and Methods

6.3.1 Cell culture and transfection:

N1E-115 mouse neuroblastoma cells (CRL-2263) were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and supplemented with 10% FBS. Cells were harvested by incubating them in Modified Puck's Saline D1 solution at room temperature until the cells detached. MCF-10A cells were cultured in MEGM medium (Lonza), MDA-MB-231 and HT-29 cells in DMEM and PC-3 cells in RPMI medium supplemented with 10% FBS. All lines were tested and were free of Mycoplasma contamination. N1E-115 cells
were transfected with X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer's instructions.

6.3.2 **Cellular protein preparation, Western blotting, Immunofluorescence and antibodies and other reagents:**

Cells were cultured to ~70% confluence and protein lysates were collected following drug treatment, transfection or otherwise for western blotting following methods described in previous publications\textsuperscript{13}. For immunofluorescence staining, cells grown on glass coverslips were fixed with cold (−20 °C) methanol or 4% paraformaldehyde (room temperature) for 10 min and blocked by incubating with 2% bovine serum albumin/PBS/0.05% Triton X-100 at 37 °C for 1h. Specific primary antibodies were incubated with coverslips for 1h at 37 °C at the recommended dilution followed by 1:2000 dilution of Alexa 488- or 555-conjugated secondary antibodies. Antibodies against γ-tubulin, α-tubulin and β-actin were from Sigma (St. Louis, MO, USA), cleaved caspase-3 was from Cell Signaling (Danvers, MA, USA). Alexa 488- or 555-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA, USA). Anti-Ki67 antibody was from Abcam (Cambridge, MA, USA). Anti-GM130 antibody was from BD Biosciences (San Jose, CA, USA). Anti-vinculin antibody was purchased from Millipore (MA, USA). Anti-lamin A/C, anti-centrin-2 and anti-CLASP1 antibodies and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CLASP1 siRNA was ordered from Origene (MD, USA). SMARTpool: ON-TARGETplus KIFC1 siRNA (Dharmacon, PA, USA) was used to knockdown HSET in N1E-115 cells.

6.3.3 **Electron microscopy:**

N1E-115 cells grown on coverslips made of Aclar film (Electron Microscopy Sciences) and were processed for electron microscopy as described in previous publication\textsuperscript{32}.
6.3.4 Neurite extension assay:

N1E-115 cells were grown on glass coverslips coated with laminin in 35-mm tissue culture dishes and treated with respective drugs diluted in serum free medium; the cells were then fixed, labeled, and visualized by immunofluorescence microscopy. Cells bearing neurite-like structures with a length of at least one cell diameter were identified by immunofluorescence microscopy using α-tubulin antibody. At least 200 cells were counted for each condition and the experiments were repeated three times. The images were taken using a Zeiss LSM 700 confocal microscope.

6.3.5 Cell-clock assay:

N1E-115 cells were grown to 60-70% confluence and then treated with either Griseofulvin, RedBr-Nos or PJ-34 for 3h and 6h. After the end of treatment, the cell clock dye (pre-warmed at 37°C) was added (150µl per well in 12-well plate) and the cells were incubated at 37°C for 1h. Dye was the washed twice with pre-warmed DMEM medium and PI was added for 15 min at room temperature and the washed twice with PBS. Fresh medium was added and the cells were imaged in bright field (to assess different phases of cell cycle) and fluorescent (red for PI) channel. Cell clock dye is a redox dye, which is readily taken up by live cells. In G1 phase, the dye in its reduced form is yellow in color, while in the intermediate state it is green (S and G2 phase) before turning dark blue in the fully oxidized form (mitosis). Micrographics taken in the bright field channel depicts cells in different cell cycle phases based on their respective colors.
6.4 Results

6.4.1 High-grade cancers show robust centrosome amplification and clustering in interphase cells unlike cultured cell lines

We first assessed if mitotic and interphase centrosome clusters are present in samples derived from high-grade carcinomas of the breast, prostate, and colon. Contrary to the notion that high-grade cancers contain relatively large proportions of mitotic cells, we found that less than 2% of cells harbored mitotic spindles in the tumor samples examined (n=8 for each tissue). To assess centrosome amplification, we counted the number of γ-tubulin dots associated with 500 nuclei in each tumor sample. In most cases, centrosomes in tumor areas appeared significantly larger than centrosomes in adjacent uninvolved tissue. Exact centrosome numbers in these enlarged centrosomal clusters were difficult to determine due to tight centrosome clustering. We therefore determined centrosomal volumes by measuring the γ-tubulin spots using the 3-D volume rendering function in the Zeiss imaging software (Axiovision LE). If the volume of a centrosome was determined to be greater than 0.76 cubic micron (maximum volume of a centrosome observed in adjacent uninvolved tissue), it was considered a case of centrosome amplification. All tissue specimens showed centrosome amplification in 60-85% of tumor cells (Fig. 6.4.1A,Bi). In order to assess centrosome clustering in interphase, we counted centrosomes and measured centrosomal volumes in 500 interphase nuclei, and nuclei with more than 2 γ-tubulin spots or at least one γ-tubulin spot with increased volume at each MTOC were considered to show centrosome clustering. More than 75% of interphase cells exhibited centrosome clustering in all cancer types examined (Fig. 6.4.1A,Bii).
Figure 6.4.1 Clinical tumors show rampant centrosome amplification and clustering in interphase cells.
A. Representative immunofluorescence confocal micrographs showing centrosome amplification and clustering status in normal adjacent (left panel) and tumor tissues (right panel) from 10
patients of each cancer type. Insets show clustered centrosomes in representative mitotic cells (top inset) and interphase (bottom inset) in tumor samples and normal centrosomes in the normal samples. White arrows depict centrosome clusters in interphase cells. Centrosomes and microtubules were visualized by immunostaining for γ-tubulin (green) and α-tubulin (red), respectively. DNA was DAPI-stained (blue). Bi-i. Quantitative bar graphs representing percentage centrosome amplification and percentage of interphase cells with amplified centrosomes that exhibit centrosome clustering, respectively in the corresponding patient tissue samples. Centrosomes were counted in interphase cells from randomly selected fields totaling at least 200 cells per sample. C. Representative immunofluorescence confocal micrographs showing centrosome amplification and clustering status during interphase in MCF-10A, MDA-MB-231, PC-3 and HT-29 cell lines. Insets show amplified and clustered/declustered centrosomes in interphase cells. Centrosomes and microtubules were visualized by immunostaining for γ-tubulin (green) and α-tubulin (red), respectively. DNA was DAPI-stained (blue). Di-ii. Quantitative bar graphs representing percentage centrosome amplification and percentage cells with amplified centrosomes that exhibit centrosome clustering, respectively in the corresponding cell lines. Centrosomes were counted in interphase cells from randomly selected fields totaling at least 200 cells per cell line. p<0.05. Scale bar, 5 µm. BC=Breast cancer, PC=Prostate cancer, COL=Colon cancer.

In contrast, 6-18% of cancer cells in culture showed mitotic spindles (data not shown), which was significantly higher than the corresponding percentage in human tumors. We found that only 5-20% of cells in cultured cell lines exhibited amplified centrosomes (Fig. 6.4.1C,Di,Dii), a lower frequency than that observed in patient tumors (Fig. 6.1Bi). We also observed that the multiple centrosomes in cell lines occurred as a juxtanuclear cluster in interphase cells (Fig. 6.4.1C). Thus, while cancer cells in culture exhibit much higher levels of mitotic activity and lower levels of centrosome amplification compared to cancer cells within patients’ tumors, cancer cells in culture and in tumors display the common features of centrosome clustering in interphase as well as in mitosis.

6.4.2 *Murine neuroblastoma cells constitute a good model system to study centrosome declustering*

To identify an ideal *in vitro* model system to study interphase-specific centrosome declustering events, we evaluated murine neuroblastoma N1E-115 cells. We found that 100% of
N1E-115 cells harbor amplified centrosomes (5-20 centrosomes per cell). We also found that the centrosomal cluster in N1E-115 cells is a mélange of single, free-standing mother and daughter centrioles and a few canonical centrosomes (data not shown). We thus wondered how these cells haul their centrosomal load through the cell cycle phases to accomplish cell division. In N1E-115 interphase cells, the multiple centrosomes localized as a distinct juxtanuclear cluster (Fig. 6.4.2A,B). However, in ~10% cells, the multiple centrosomes showed significant scattering and this feature correlated with chromatin condensation and absence of a mitotic spindle. Lamin A/C immunostaining showed that cells with loose centrosome clusters still had an intact nuclear membrane. Thus, these cells were confirmed to be in prophase (Fig. 6.4.2A,B). About 30% of mitotic cells were in prometaphase (i.e., they lacked a nuclear membrane) and possessed multipolar spindles with multiple MTOCs at each spindle pole. Metaphase cells, by contrast clustered supernumerary centrosomes into two polar groups to generate a pseudo-bipolar spindle (Fig. 6.4.2A,B). The centrosomes at the poles of the spindle were often arranged in “ring-like” or “V-shaped linear” configurations (Fig. 6.4.2A). Only ~20% cells displayed equal centrosome counts at the two spindle poles. CREST antibody labelling revealed that in metaphase cells, the kinetochores did not line up immaculately along the spindle equator perhaps due to widespread merotelic attachments (Fig. 6.4.2C). Centrosomes remained clustered at the two spindle poles through anaphase and the occurrence of occasional lagging chromosomes indicated chromosome missegregation (Fig. 6.4.2D). Telophase was marked by nuclear envelope reassembly around the decondensing chromatin and the inheritance of a juxtanuclear centrosomal cluster by each daughter cell (Fig. 6.4.2A,B). These observations indicated that murine N1E-115 cells are a great model system to study centrosome clustering and declustering since they show both interphase and mitotic centrosome clustering similar to patient tumor cells.
Figure 6.4.2 Cell cycle phase characterization of N1E-115 cells.
A. Representative immunofluorescence confocal micrographs depicting centrosome status in all cell cycle phases of N1E-115 cells. N1E-115 cells in interphase possess an enormous number of centrosomes as evident by γ-tubulin immunostaining (green). We acquired images as z-stacks with the slice interval of 0.40 μm. Z-stack slices encompassing the entire depth of the cell were then merged and γ-tubulin positive spots were counted in interphase cells from randomly-
selected fields totaling 200 interphase cells. B. Representative immunofluorescence confocal micrographs showing lamin A/C staining (red) across all cell-cycle phases to visualize nuclear membrane in order to distinguish interphase declustering from prophase scattering of centrosome cluster. C. Confocal micrograph of a metaphase cell stained with CREST antibodies (white), antibodies against α-tubulin (red) and γ-tubulin (green), and DAPI (blue) to detect microtubule-kinetochore attachments and DNA in a pseudobipolar mitotic spindle. D. Representative micrograph of an anaphase cell immunostained for α-tubulin (red), γ-tubulin (green) and DAPI (blue) showing lagging chromosomes. Scale bar, 5 µm.

In order to probe the mechanisms facilitating interphase clustering in N1E-115 cells, we examined the involvement of two major microtubule motors (HSET and dynein) in centrosome clustering in interphasic and mitotic N1E-115 cells. We found that siRNA knock down of the kinesin-14 protein KifC1/HSET resulted in robust mitotic declustering generating ~65% multipolar mitotic cells, but negligible interphase declustering (15% as compared to 12% in vehicle treated controls) (data not shown). However, dynein inhibition by ciliobrevin treatment demonstrated substantial scattering of interphase centrosomal clusters (~50%) and considerable mitotic declustering (~35%) (data not shown). Based on these data, it seems that while HSET is crucially involved in mitotic centrosome clustering, dynein plays the major role in maintaining the centrosomal cluster during the subsequent interphase.

6.4.3 Centrosome declustering agents disperse interphase clusters and set the stage for a catastrophic mitosis

Given the limited mitotic populations in human cancers, centrosome declustering during mitosis alone would fail to achieve sufficient elimination of cancer cells. On the other hand, interphase declustering may not only prime the cell for catastrophic mitosis but also ensure disruption of interphase-specific cellular processes that undergird migration. Thus, we investigated how declustering agents affect centrosome clustering during interphase. We tested three declustering drugs (RedBr-Nos, Griseofulvin and PJ-34)\textsuperscript{13-17} and compared them with
Paclitaxel, a tubulin polymerizing drug. RedBr-Nos, Griseofulvin and Paclitaxel are known to bind tubulin\textsuperscript{18-20} but PJ-34 is a poly-ADP-ribose polymerase (PARP) inhibitor with no known tubulin binding property. However, they share common phenotypes such as mitotic arrest and multipolar mitoses\textsuperscript{13-17,21}. We found N1E-115 cells to be more sensitive to these drugs compared to other cancer cell lines (for instance, MDA-MB-231, HeLa) with IC\textsubscript{50} values ranging between 0.05 µM for Paclitaxel and 25 µM for Griseofulvin (data not shown). To evaluate their effect on interphase clustering, we treated N1E-115 cells with drugs at their respective IC\textsubscript{50} concentrations for 0, 3, 6, 9h and co-immunostained for γ-tubulin and α-tubulin to evaluate centrosomal spread and microtubule nucleation status, respectively (Fig. 6.4.3A). RedBr-Nos and Griseofulvin inflicted more severe interphase declustering compared to PJ-34 and Paclitaxel. We also verified the cell cycle phases via lamin A/C immunostaining to distinguish interphase declustering events from prophase centrosomal spread. To quantitate the spread of the interphase centrosomal cluster, we generated a 3-D reconstruction of z-stack images of 25 randomly-selected interphase cells from the 6h treatment group of each drug. By defining an ROI (region of interest) around the interphase centrosomal cluster, we calculated volume of the cluster spread using Volocity software as shown in Fig. 6.4.3Bi. Likewise, defining an ROI using the cell periphery provided the cell volume. We defined the interphase declustering index (DI) for each drug as the ratio of the average volume of clusters to the average volume of the corresponding cell. Quantitative evaluation of DI revealed RedBr-Nos as the strongest declustering agent (DI=0.36), followed by Griseofulvin (DI=0.28) and PJ-34 (0.14). Paclitaxel showed the least declustering effect with a DI of 0.08 as compared to 0.02 in control cells (Fig. 6.4.3Bii). We also found that dispersal of the interphase centrosome cluster precipitated multipolar mitoses in the treated cells (Fig. 6.4.3C). Again, the proportion of multipolar cells was higher in RedBr-Nos and Griseofulvin-
treated cells as compared to cells treated with PJ-34 and Paclitaxel, which mirrored the trend in interphase declustering (Fig. 6.4.3C). These observations suggest that interphase declustering of centrosomes compels cells into catastrophic multipolar mitoses.

**Figure 6.4.3 Interphase declustering induced by centrosome declustering agents.**

A. Confocal micrographs showing interphase declustering induced by 6h treatment with RedBr-Nos (10 µM), Griseofulvin (50 µM), PJ-34 (25 µM) and Paclitaxel (0.1 µM). Percentages indicate proportion of interphase cells with declustered centrosomes. Bi. 3-D representation and quantitative volume analysis of control and drug-treated interphase cells using Veloctiy 6.3 software. Cells were co-immunostained for Lamin A/C (red) and γ-tubulin (green) and z-stacks were acquired with a 0.35 µm z-step. Z-stack slices were then used to construct a 3-D image and ROIs were defined to generate declustering index (DI) measurements. Bii. Quantitative bar graph
representing the DI of the four drugs. p<0.05. 

**C.** Confocal micrographs showing spindle multipolarity induced by 18h treatment with declustering agents RedBr-Nos (10 µM), Griseofulvin (50 µM), PJ-34 (25 µM) and Paclitaxel (0.1 µM). Cells were co-immunostained for α-tubulin (red) and γ-tubulin (green). Percentages indicate proportion of mitotic cells with declusted centrosomes. 

**D.** Confocal micrographs showing Golgi dispersal concomitant with interphase declustering upon 6h treatment with all the four declustering agents at stated concentrations. Cells were co-immunostained for GM130 (red) and γ-tubulin (green). DNA was DAPI-stained. Scale bar, 5 µm.

### 6.4.4 Centrosome declustering in interphase disrupts Golgi coalescence and inhibits migration

The Golgi, which is primarily responsible for posttranslational modification and protein sorting, also functions as a MTOC\(^{22}\). It has been hypothesized that supernumerary centrosomes may better organize the Golgi to enhance directional cell migration\(^{23}\). We therefore investigated what happens to the Golgi upon declustering drug-induced dispersal of the interphase centrosomal cluster. We co-immunostained drug-treated cells for GM130 (a cis-Golgi matrix protein crucial for maintaining its structure) and γ-tubulin. Following treatment with declustering drugs, the interphase Golgi complex fragmented and the distribution of Golgi fragments closely mimicked scattering of the centrosomal cluster, with the most robust effect seen with RedBr-Nos and Griseofulvin (Fig. 6.4.3D).

Research suggests that Golgi-derived microtubules are not sufficient to preserve cell polarization; instead, they need to act in concert with the centrosome to establish and maintain cell polarization\(^{24}\). In cancer cells harboring a supercentrosomal cluster, we predict that disrupting the cytoskeletal and organellar framework organized by a strongly polarizing supercentrosomal cluster will present a setback to the mechanical thrust that such a cluster can empower a migrating cell with; this in turn, we predict, will lead to impaired directional migration. As a surrogate for the polarization that underlies directional migration, we decided to examine neuritogenesis, a process in nerve cells involving the extension of polarized, elongated
neurites. N1E-115 cells usually extend only one major neurite per cell, which can vary in length from 5 to 500 µm. The growth cones of the neurites serve as primary focal points of motility. We evaluated the effect of declustering agents on cell motility by assessing the length and frequency of neurites formed in a serum-free medium on a laminin-coated surface. Neurite growth under these conditions is linear for up to 24h, reaching a maximum around 36-48h after plating. Phase contrast imaging showed presence of several elongated (10-200 µm long) neurites upon 48h serum starvation (Fig. 6.4.4Bi). We observed 70-80% inhibition of neurite extension when treated with RedBr-Nos and Griseofulvin and moderate inhibition with PJ-34 and Paclitaxel treatment (Fig. 6.4.4A,Bii). Confocal imaging confirmed that inhibition of neurite formation was accompanied by dispersal of the interphase centrosome cluster, which is normally situated near the base of the tubulin-rich neurite shaft (Fig. 6.4.4C).
Figure 6.4.4 Inhibition of neuritogenesis by centrosome declustering agents.

A. Phase contrast images of N1E-115 cells in serum-starved (SS) medium showing neurite formation after 48h of SS or with RedBr-Nos (5 µM), Griseofulvin (10 µM), PJ-34 (10 µM) and Paclitaxel (0.05 µM) treatment. Scale bar, 10 µm.

B. ii. Quantitative bar graphs representing average length of neurites and percent population of cells showing neurite length >10 µm, respectively. 100 cells were counted in each case. p<0.05.

C. Confocal micrographs showing neurite outgrowth after 48h without SS, with SS, or SS along with drug treatment, respectively. Cells were co-immunostained for α-tubulin (red) and γ-tubulin (green). DNA was DAPI-stained.

D. i. Immunoblot showing CLASP1 expression levels in control and CLASP1 siRNA-transfected N1E-115 cells. ii. Confocal micrographs showing Golgi network immunostained for GM130 (red) and centrosome cluster immunostained for γ-tubulin (green) in control and CLASP1 siRNA-transfected N1E-115 cells. iii. Confocal micrographs showing neurite outgrowth in control and CLASP1 siRNA-transfected N1E-115 cells.

E. Confocal micrographs showing vinculin localization during neurite outgrowth after 48h without SS, with SS, or SS along with drug treatment, respectively. Cells were stained for F-actin using rhodamine-phalloidin, immunostained for vinculin (green) and DNA was DAPI-stained. Scale bar, 5 µm.
In order to establish whether Golgi-dependent vesicular trafficking lies downstream of interphase centrosome clustering during cell polarization and neuritogenesis in N1E-115 cells, we studied the effect of centrosome declustering-independent Golgi scattering on neuritogenesis. To accomplish this, we used CLASP1 siRNA to disrupt the Golgi-nucleated microtubules (Fig. 6.4.4Di), thus disarraying the directionality of post-Golgi vesicular trafficking but leaving the centrosome cluster intact, and evaluated whether these cells can generate neurites. We observed ~50% Golgi scattering upon CLASP1 knockdown (Fig. 6.4.4Dii). We observed that cells with CLASP1 siRNA formed significantly fewer neurites compared to control cells (Fig. 6.4.4Diii, data not shown). This observation suggests that (i) disruption of Golgi network impedes Golgi polarization-dependent neuritogenesis, and (ii) Golgi complex integrity and polarized post-Golgi trafficking lie downstream of interphase centrosome clustering.

The spatio-temporal arrangement of Golgi apparatus serves as geometrical regulator of cell migration as well as neurite extension. Thus, we wanted to determine if Golgi disruption upon CLASP1 knockdown affects cell shape and cell adhesion, modulation of which are crucial for cell migration as a precursory step for neurite extension in N1E-115 cells. We observed significant cell morphology changes shifting from majorly mesenchymal-like cell shape in cells transfected with control vector to largely amoeboid-like and more “rounded” cell shape in CLASP1 knockdown cells. This shift in cell morphology indicates changes in cell-substrate adhesion properties as a result of Golgi dispersal, which was confirmed by the reduction in vinculin localization at distinct adhesion focal points in CLASP1 siRNA cells (data not shown).

Vinculin stabilizes cell-substrate contacts in neuronal cells undergoing neuritogenesis\textsuperscript{25}, and activation by actin-binding proteins mobilizes vinculin to focal adhesions\textsuperscript{26, 27}. We therefore determined the localization of vinculin in the neurite extensions and the effect of declustering
agents on its localization. To this end, we immunostained cells for vinculin and stained F-actin using rhodamine-phalloidin. Cells in serum-supplemented medium showed vinculin localization at focal adhesions with very little internalized vinculin. Upon serum starvation for 48h, most of the vinculin was localized to the neurite growth cones. However, upon treatment of serum-starved cells with RedBr-Nos and Griseofulvin, we observed complete internalization of vinculin and complete loss of focal adhesion points. The observed effect was less severe with PJ-34 and Paclitaxel (Fig. 6.4.4E). Centrosome declustering drugs thus impair cell polarization and neurite formation and the localization of vinculin, a key player in establishment of cell-substrate contacts.

These observations underscore the immense clinical potential of centrosome declustering as a selective therapy for cancer cells harboring excess centrosomes, without affecting cells with normal centrosome content.

6.4.5 Inhibition of migration results in interphase cell death or pushes cells into catastrophic mitosis

Several studies suggest an intrinsic, inverse relationship between cell migration and cell proliferation. This concept that cells exist in mutually-exclusive cellular states that either permit motility or mitotic activity is evidenced by numerous in vitro and in vivo studies and is referred to as “Go-or-Grow”. We thus explored whether inhibiting migration via declustering drug treatment of serum-starved (SS) N1E-115 cells enhances proliferation (indicated by Ki67 nuclear immunostaining) or induces apoptosis (indicated by cleaved caspase-3 immunostaining). We found a high proportion of Ki67-positive cells when treated with RedBr-Nos (58%) and Griseofulvin (45%), followed by PJ-34 (29%) when compared to negligible number of Ki67-positive SS N1E-115 cells, which should predominantly be in G0 of the cell cycle (Fig. 6.4.5,
These data suggest that declustering drugs cause more cells to enter the cell cycle under conditions of serum-starvation. We also wanted to explore whether apoptosis is induced by interphase declustering and whether any induced cell death depends on the cells’ passage through mitosis. Cleaved caspase-3 staining in N1E-115 cells upon treatment with the three drugs for 9h (a time point at which the vast majority of cells were in interphase; data not shown) revealed a higher proportion of caspase-3 positive interphase cells in drug-treated cultures compared to untreated controls, indicating significant induction of cell death during interphase (Fig. 6.4.5, bottom panel). Interphase-specific cell death was confirmed with a cell-clock assay (data not shown). These observations suggest that disrupting the supercentrosomal cluster during interphase in N1E-115 cells (a) induces interphase catastrophe, and (b) pushes cells into a proliferative mode leading to a catastrophic mitosis. These data thus support the notion that centrosome declustering drugs launch a two-pronged attack on supercentrosomal cells.

**Figure 6.4.5 Inhibited migration induces interphase cell death or pushes cells into catastrophic mitosis.**

A. Confocal micrographs showing proliferative cells with 24h of SS, or SS along with RedBr-Nos (5 µM), Griseofulvin (10 µM), and PJ-34 (10 µM) treatment, respectively. Cells were co-immunostained for Ki67 (red) and γ-tubulin (green). Percentages show proportion of Ki67-positive cells. Scale bar, 5µm. Confocal micrographs showing cells undergoing apoptosis with 9h of SS, or SS along with RedBr-Nos (5 µM), Griseofulvin (10 µM), and PJ-34 (10 µM) treatment, respectively. Cells were co-immunostained for cleaved caspase-3 (red) and α-tubulin (green). Percentages show proportion of cells that stained positive for cleaved caspase-3. Scale bar, 10µm. B. Quantitative bar graphs representing percentage Ki67 and caspase-3 positive cells when treated with respective drugs. 200 cells were counted in each case. p<0.05.
6.5 Discussion

Majority of cancer patients succumb to cancer due to metastases for which effective therapeutic options are currently lacking. Cell migration and invasion are the key cell biological processes that underlie metastatic dissemination of cancer cells. With the recent realization that patient tumors are slow growing with doubling times ranging between 100 to 700 days, the glory of mitosis as a target has faded. Most anti-mitotic drugs have failed so far in clinical trials either due to limited efficacy, since most cells in patients’ tumors are not mitotic, or excessive toxicity; thus interphase is a more promising chemotherapeutic target.

Recent studies have provided in vitro evidence that centrosome amplification can cause oncogene-like effects in promoting cellular invasion in mammary epithelial cells. These findings assert that structural alteration of the cytoskeleton via centrosome amplification confers transformation potential to normal epithelial cells and is directly responsible for tumor initiation and progression. Our study herein is the first to demonstrate that interphase cancer cells in patients’ tissues organize their excessive centrosomal load in the form of a juxtanuclear supercentrosomal cluster. This tight cluster is maintained throughout interphase and disperses only transiently during prophase followed by reclustering. We show that untimely dispersal of the supercentrosomal cluster in interphase drastically impacts cytoskeletal and organellar organization; in particular, the Golgi fragments and each dispersed centrosome carries with it a group of associated Golgi fragments. As a consequence, the cells are no longer able to produce neurite extensions and establish proper focal contacts with the substrate, as needed for directional migration. Interphase clustering of supernumerary centrosomes is thus a cancer-specific trait that may help cancer cells survive and migrate. We assert that a powerful strategy to cripple the
migratory agenda of cancer cells is to disrupt the centrosomal cluster by using centrosome declustering agents.

The importance of interphase clustering to cancer cells is spotlighted by the dire consequences of disrupting the interphase centrosomal cluster. It has been established that polarity of the Golgi complex and directionality of Golgi-nucleated microtubule arrays are crucial for directional cell migration. The correct orientation and positioning of the Golgi apparatus is regulated by interplay of various factors, including both centrosome- and Golgi-derived microtubules, and the binding of AKAP450 to GM130, gamma-TuRC and dynein-dynactin complex. Our data confirms the scattering of GM130 accompanied by centrosomal cluster scattering upon action of declustering agents, indicating possible scattering of AKAP450 as well. Thus, disrupting the centrosomal cluster leads to concurrent scattering of the Golgi apparatus as a result of which Golgi-derived microtubules and post-Golgi vesicular trafficking are no longer focused towards the leading edge, which may have a dramatic effect on directional cell migration.

Thus, centrosome declustering drugs launch a two-pronged offensive on supercentrosomal cancer cells in that they not only scatter the centrosomes through the cytoplasm and profoundly disrupt the Golgi network to impede cell migration in interphase, but also effectively trap cancer cells in a non-resolvable state that culminates in spindle multipolarity and metaphase catastrophe in mitosis. We are confident that the observed phenotypes are triggered by centrosome cluster dispersal and not due to the drugs used, since the declustering drugs in our study function through very different mechanisms and yet produce similar phenotypes.
Our study demonstrates that mouse neuroblastoma cells N1E-115 are an excellent test-bed for studying the mechanisms and effects of centrosome clustering and declustering. These cells, unlike other cell lines, have 100% centrosome clustering in interphase and ~90% "pseudobipolar" spindle formation in mitosis. A “good” declustering drug should be able to scatter its megacentrosomal cluster into an unrestricted pool of centrioles in this cell line, consequently generating excessive spindle multipolarity and severe, death-inducing aneuploidy in daughter cells. The DI as described for N1E-115 cells in our study can facilitate quantitative comparison of the efficacy of putative declustering agents. Based on our data, RedBr-Nos and Griseofulvin showed more dramatic effects on centrosome declustering and inhibition of neurite formation as compared to PJ-34 and Paclitaxel. In sum, our findings reveal the previously under-appreciated aspects of the actions of centrosome declustering drugs, their potential application as anti-metastatics and the importance of interphase as a chemotherapeutic target.

6.6 References


7. A NOVEL METRIC THAT QUANTITATES THE KINETICS OF CELL CYCLING AMONG PROLIFERATING TUMOR CELLS UNCOVERS TUMORS’ CHANGING AGENDA AND IMPROVES BREAST CANCER PATIENT STRATIFICATION

7.1 Abstract

Ki67 Index (KI) and mitotic index (MI) are widely used proliferation markers with established prognostic value in breast cancer diagnostic pathology. While KI is evaluated immunohistochemically and reported as a percentage, determination of MI, which is conveyed as an absolute count of total mitotic cells in 10 high-power fields, relies on visual recognition. The influence of subjectivity coupled with disparate measurement scales, and lack of rational integration of these mutually-inclusive indices, leads to loss of information that could strengthen the prognostic accuracy of tumor grade. Pathology reports from 1611 breast carcinoma cases at Northside Hospital, Atlanta, GA were analyzed retrospectively. KI and MI were brought on the same measurement scale by deriving a Ki67-Adjusted Mitotic Score (KAMS). Findings from our data mining study were validated in paraffin-embedded breast tumor specimens (n=233) using a first-of-its-kind multicolor indirect immunofluorescence confocal imaging technique that enabled accurate determination of the proportion of mitotic cells amongst the proliferating population (i.e., M-to-P Ratio) by quantifying Ki67-positive and mitotic (phospho-histoneH3-positive) cells on the same scale from the same field-of-view. Retrospective data analysis revealed that although KI and MI both increased with tumor grade, the rate of increase in MI was much slower than KI. Consequently, KAMS decreased across grade, suggesting a striking grade-wise decrease in the proportion of actively dividing cells amongst Ki67-positive proliferative population. Intriguingly, lower M-to-P ratios in Nottingham Grade II and III tumors correlated with poorer
overall survival. The strikingly lower mitotic propensities observed in poorly-differentiated breast tumors as compared to those in well differentiated tumors offer unique teleological insights into tumor evolution and spotlight the dynamic nature of tumor agendas, drifting from a “mitosis-heavy” program (that fosters rapid generation of clonal diversity) to a “migration-focused” one that supports metastatic dissemination. Incorporation of our new metric into the Nottingham Grading System (NGS) or a KI-based stratification system, and Cox proportional hazards analyses of overall survival data showed that integrating KI and MI enables superior patient stratification than the traditionally-used NGS or KI-based systems. Here we uncover a previously unrecognized dynamic relationship between KI and MI by pioneering a novel histological metric, Mitosis-to-Proliferation Ratio (M-to-P Ratio; also known as KAMS (Ki67-Adjusted Mitotic Score) for retrospectively analyzed data) that rationally integrates KI and MI on the same scale of measurement to provide new risk-predictive information that cannot be gleaned when KI and MI are extracted independently from non-overlapping fields. We report for the first time that M-to-P ratio decreases across breast cancer grades, which, in essence, unmask the hitherto unappreciated fact that the proportion of mitotic cells amongst Ki67-positive cells decreases with advancing tumor grade despite clear grade-wise increases in KI and MI. In other words, proliferating cells in high-grade tumors tend to spend increasing durations of time lingering in interphase compared to proliferative cells in low-grade tumors. Our integrated metric that quantifies the kinetics of cell cycling within the proliferative population may thus reveal a new layer of valuable and clinically-actionable risk-predictive information for deeper patient stratification to enable optimal therapeutic decision making.
7.2 Introduction

Therapeutic decision-making for personalized management of breast cancer relies on patient stratification based on the risk conferred by clinicopathologic factors. The prognostic and predictive molecular markers commonly used for assessing the risk associated with a breast tumor or its “aggressive potential”, include the status of the cell proliferation marker Ki67, estrogen receptor (ER), progesterone receptor (PR), extent of amplification of the EGF receptor Her2, and mitotic index (MI) of the tumor. Of these markers, MI is an integral element of the Nottingham Grading System (NGS), which is a modification of the Scarff-Bloom-Richardson tumor grading system.\textsuperscript{1} The NGS provides clinically important prognostic information about breast tumor samples by combining analysis of the extent of glandular differentiation, nuclear pleomorphism and mitotic activity present in the tumor sample.\textsuperscript{2} Tumor grading in this method involves microscopically evaluating three histological parameters and assigning a score of 1 to 3 for each of them: tubule formation (<75% = 1, 10% to 75% = 2, and >10% = 3), nuclear pleomorphism (none = 1, moderate = 2, and marked = 3), and mitotic activity found in 10 high-power fields (HPF), based on a HPF size of 0.196 mm\textsuperscript{2} (<7 mitoses = 1, 7 to 14 mitoses = 2, and >14 mitoses = 3). Summation of the three scores thus obtained determines the placement of the tumor into one of three Nottingham Grades: combined score of 3, 4, or 5 = grade 1; combined score of 6 or 7 = grade 2; and combined score of 8 or 9 = grade 3.

Since a tumor’s proliferation kinetics has a crucial bearing on its clinical course, a great deal of emphasis has been placed on quantifying the rate of tumor proliferation as an adjunct to diagnosis. So in addition to quantitating mitotic counts for histological grading, pathologists have been staining the Ki67 antigen that labels cells in the G1, S, G2 and M phases of the cell cycle to enable easy measurement of the proliferating cell population within tumors.
Nuclear Ki67 positivity is defined as the percentage of stained carcinoma cells. Ki67 protein is present during all cell cycle phases (G1, S, G2 and M) and is strictly a cell proliferation marker. Ki67 is highly recommended for distinguishing between “luminal A” and “luminal B” subtypes in breast cancer patients. However, clinical arena still lacks consensus on standard Ki67 assessment protocols and cut-off values, thus making it a rather poorly reproducible index of tumor proliferation. Various studies have established statistically significant association between Ki67 and breast cancer prognosis except that the cut-offs used in the studies to categorize “high” and “low” Ki67 groups varied from 1% to 28.6%, indicating the unreliability of its clinical significance. Owing to the lack of clarity on how Ki67 score should influence clinical decisions, it was not included in the routine decision-making in breast cancer patients, until recently, even though high Ki67 levels are often associated with worse outcomes.

In 2010, at the “International Ki67 in breast cancer working group” headed by DR. Dowsett and Dr. Hayes, investigators came to a consensus regarding the use of Ki67 as a standard marker for monitoring tumor proliferation. However, inconsistencies in Ki67 staining assessment and interpretation were also highlighted. In 2011, the same group presented a standard guideline for consistent and improved assessment of Ki67 to enable its utility in breast cancer management.

Apart from their independent prognostic value, the precise relationship between KI and MI is also inadequately understood and has been found to be context-dependent. Few studies reported little or no correlation between KI and MI in small cell lung carcinoma and squamous cell carcinoma, whereas other studies showed inverse relationship between the two. A larger number of studies showed positive correlation between KI and MI, with a strong relation in breast and non-small cell lung carcinomas and a relatively weaker relation in colorectal
adenocarcinomas, neuroendocrine neoplasms of pancreas and gastrointestinal tract. A number of studies suggest a tight correlation between KI and MI in well- and moderately- differentiated tumors and insignificant correlation in poorly differentiated tumors. In reality, there exists an enormous void in the current understanding of KI and MI as autonomous prognosticators and precise mechanics of their relationship in the context of tumor development.

There is scope for improving the prognostic accuracy of NGS as evidence suggests that patients are still being over or under-treated. Hence there is a need to refine the NGS and enhance its prognostic accuracy by identifying quantifiable biomarkers for breast tumors that (i) can discriminate more sharply the risk posed by breast tumors, (ii) can be accurately and reliably determined via a clinically facile method, (iii) are robust and applicable across the wide spectrum of clinical behaviors observed in various subtypes of breast carcinomas, and (iv) yield more accurate patient stratification.

To improve accuracy of NGS, one would need to improve the accuracy in determining its constituent parameters. We will briefly mention two kinds of errors – firstly, two different fields as well as two different scales of measurement. Second error is mis-estimation due to visual recognition, subjectivity (intra- and inter-observer, different regions chosen-different cellularity, etc. Moreover, visual recognition is time consuming. Another issue is with current diagnostic practices: MI and KI are being considered as independent entities with no connection to each other. In reality, mitosis is a cell cycle phase nested within the proliferative cycle. Since they are currently perceived as disparate entities, an integrated or unified view is impossible which might have offer additional layer of risk predictive and stratification information.
To test this concept, we have carried out a retrospective study of clinical data and showed that a novel metric that rationally integrates KI and MI to estimate the total number of mitotic cells among Ki67-positive cells in a tumor sample (i.e., the Ki67-adjusted mitotic score or KAMS) reveals the dynamic agenda of an evolving tumor. We then extracted KI and MI from the same field and evaluated whether the mitosis-to-proliferation ratio (M-to-P ratio), which reflects the kinetics of cell cycling within the proliferative population of tumor cells has the capacity to provide deeper risk-segmentation of patients into prognostically meaningful subgroups for more optimal therapeutic decision-making.

7.3 Materials and Methods

7.3.1 Data mining:

Clinical records of 4342 breast cancer patients, who were diagnosed with the disease between 2005 and 2009, were mined from Northside Hospital, Atlanta. Histologic grading performed by pathologists at Northside Hospital involved an evaluation of three parameters and assignment of a score of 1 to 3 for each parameter: tubule formation (>75% = 1, 10% to 75% = 2, and <10% = 3), nuclear pleomorphism (none = 1, moderate = 2, and marked = 3), and mitotic activity within 10 high-power fields (HPFs), based on a HPF size of 0.183 mm² (<7 mitoses = mitotic score 1, 7 to 14 mitoses = mitotic score 2, ≥ 14 mitoses = mitotic score 3). Amongst these patients, 2731 whose records were missing information regarding either Ki67 index (KI), Mitotic index (MI), nuclear differentiation, or tubular formation were excluded from our analysis. The clinical records of the remaining 1611 patients who met all the inclusion criteria were then used for analyses.
7.3.2 *Ki67 adjusted mitotic score (KAMS) formulation:*

Due to mitotic index being recorded as a monotonic categorical variable it was difficult to relate it to Ki67 values, which were recorded as a percentage of total cells. Therefore, some assumptions had to be made in order to derive the percentage of mitotic cells in each patient. First, we evaluated 10 HPFs in at least 5 patient samples and found that on average, 10 HPFs have ~500 cells. Second, we converted the patients’ mitotic scores into mitotic cell counts as follows: For patients with mitotic scores 1 and 2, we assumed mitotic cell counts of 3.5 and 11 (the average cell count value of those score ranges), respectively. For patients with mitotic score of 3, we assumed a mitotic cell count of 15 (which is the floor value for the mitotic score 3 category). These mitotic cell counts gave us an estimation of the number of mitotic cells out of 500 cells (10 HPFs), thus providing us the percentage of cells undergoing mitosis. Ki67 adjusted mitotic score (KAMS) for each patient was then calculated simply as the quotient of the percent mitotic cells divided by the percent Ki67-positive cells.

7.3.3 *Statistical Analysis:*

Differences between baseline results were established using a one-way analysis of variance (ANOVA) alongside a post-hoc Tukeys range test to indicate any significant difference between specific groups. Survival curves were obtained via the Kaplan-Meier method with significance determined using the log-rank test. Survival time was measured from the initial diagnosis to either an event (death) or to the final follow up (censor) and was thus an indicator of overall survival. To obtain hazard ratios and the fit statistics, we employed the Cox proportional hazard model. For categorical variables, the lowest risk group was used as the reference to that parameter’s hazard. For ideal thresholds, the FINDCUT macro developed by Jayawant N. Mandrekar et al. from Mayo Clinic (http://www2.sas.com/proceedings/sugi28/261-28.pdf) was


used, which identifies the optimal cut off point for a continuous variable that predicts time to event outcomes, in our case KAMS and M-to-P ratio.

Using a macro by Mithat Gönen (% C-index), based on work done by Herrel et al. (1982), we are able to determine the concordance index (C-index) for our models, with censored outcomes. The concordance index for censored data is the summation of all concordant data pairs divided by the total number of verifiable pairs (as in some cases, due to censored data, it is impossible to determine). With concordant pairs being indicated by proper model prediction segmentation of events as well as the time to event. In order to compare multiple model c-indices for difference in significance, we utilized a 100x bootstrap method where the model was tested on 40% of the bootstrap sample and then validated on the remaining 60%. The mean C-indices could then be compared with a student t-test.

7.4 Results

7.4.1 Quantitation of mitotic figures from H&E stained slides underestimates mitotic population

KI, a universal independent prognostic marker, and MI, an integral element of the currently used Nottingham Grading System (NGS) are integral to the assessment of breast tumor metastatic risk. Despite the knowledge that mitotic cells are a subset of the Ki67-positive proliferating cell population, the precise relationship between KI and MI has remained poorly defined and deemed context-dependent. KI and MI have also not achieved sufficient reproducibility and reliability to enable accurate prediction of disease prognosis and aggressiveness. We believe that inaccuracies in their determination might be one of the crucial factors contributing to the limited prognostic accuracy of these markers. In current clinical
practice, while KI is evaluated at low magnification as percentage of nuclei in a tissue section that stain positive for the Ki67 antigen when at least 500-2000 total cells are counted, MI is determined via visual recognition of mitotic cells in 10 high power fields (HPFs) in an independent H&E-stained tissue section. The independent determination of these indices from different sections and the use of disparate scales for their evaluation (i) overlooks the fact that mitotic cells comprise a subset of cycling cells; (ii) makes a direct comparison of KI and MI impossible (Fig. 7.4.1A, middle panel) and (iii) precludes evaluation of mitotic propensity and the cell cycling kinetics of the proliferative population within the tumor. Essentially, we envision that the proportion of mitotic cells amongst the proliferative population within a tumor provides a measure of the risk associated with the tumor due to erroneous mitoses. This “dangerous” fraction of proliferating cells could potentially be accurately quantitated from a simultaneous visualization of both mitotic and Ki67-positive cells in the same field (Fig. 7.4.1A, right panel).

Indeed, the determination of MI is a time-consuming process that requires skilled observers (Hall and Levison, 1990) since discerning a prophase cell from a pyknotic or apoptotic cell is challenging in H&E-stained sections, even at the relatively high magnifications required to unequivocally identify mitotic phases. A commonly used marker to identify M-phase cells is phosphorylated histone H3 (p-H3). Histone H3 becomes phosphorylated on serine 10 upon chromosome condensation during prophase (Chadee et al., 1999; Strahl and Allis, 2000; Cunningham, 2002), and remains phosphorylated until telophase, thus making p-H3 a very specific and reliable mitotic marker representing all phases of mitosis including prophase. In order to assess the value of p-H3 for determining the mitotic score by immunocytochemistry in paraffin sections, we stained 45 paraffin-embedded breast tumor sections with either H&E or anti-p-H3 antibody and carried out a quantitative head-to-head comparison of mitotic scores for
the same pre-marked areas in the two sets of slides. Three different pathologists scored tumor tissues for mitotic cells in a blinded manner. Mitotic score for each case was compared between H&E and p-H3-stained tissues by counting nuclei in 10 HPFs from pre-marked matched areas on each tumor tissue pair (Fig. 7.4.1B). We found considerably higher and more reproducible mitotic scores as assessed by p-H3 staining in comparison to direct counting of mitotic figures in H&E stained sections (Fig. 7.4.1Ci). Even cells in different mitotic stages were easily identified by p-H3-staining compared to H&E stain; prophase cells, in particular, were more readily identified by p-H3 staining. We observed average mitotic scores via p-H3 staining to be higher by an average of 46.6% for three pathologists (p<0.0001) (Fig. 7.4.1Ci). Mitotic counts using H&E staining showed a moderate positive correlation with counts from p-H3 stain (r=0.35, p<0.001). Moreover, the better contrast in p-H3 and easier recognition of mitotic figures enabled more rapid determination of mitotic scores in p-H3-stained slides (Fig. 7.4.1Cii) demonstrating the usefulness of p-H3 as a reliable and efficient mitotic marker. The average time taken by each pathologist in scoring p-H3 slides was ~37% lower than H&E slides, irrespective of the individual scoring time differences between the 3 pathologists.

Next, we used Intraclass correlation coefficient (ICC) to assess the consistency of measurements made by the three pathologists. We found that there was a significant increase in agreement among the three pathologists when evaluating mitotic scores using p-H3 staining (ICC=0.57) compared to mitotic scores using H&E staining (ICC=0.38) (p<0.05). These data underscore that p-H3 stain significantly increases inter-observer reproducibility than H&E in evaluation of MI, thus establishing the superiority of p-H3 for mitotic cell quantitation.
Figure 7.4.1 Schematic representing divergent perspective of a pathologist and a researcher regarding an actively dividing cell.

A. Pathologists view Ki67-positivity and mitosis as two mutually exclusive events in cell cycle, whereas a researcher views mitosis as a subset of the full cycle of a proliferating or Ki67-positive cell. B. Micrographs showing various stages of mitosis in an H&E stained paraffin embedded breast tumor tissue section. Scale bar, 20μm. C. Bar graphs representing average mitotic counts determined by counting mitotic figures from H&E-stained or p-H3-stained slides, by each of the three pathologists. Cii. Box and whisker plot representing the average time take by the three pathologists to score H&E-stained or p-H3-stained slides. Ciii. Scatter plot showing the difference in the ICC of p-H3-based versus H&E-based counting, with the confidence intervals. (t-test p < 0.05).
7.4.2 *The relationship between KI and MI is a highly dynamic one that shows grade-wise variations*

In order to revisit the relationship between KI and MI across Nottingham Grades I, II and III (termed NGI, NGII and NGIII, respectively), we analyzed clinical data from 1611 breast cancer patients from Northside Hospital, Atlanta, for whom complete information regarding grade, KI and MI was available (clinicopathological characteristics of patient cohort are described in Table 1). Per standard clinical practice at this hospital (as well as in two other major hospitals in the greater Atlanta area), we found that tumor samples were categorized into 3 groups based on their mitotic scores (Mitotic Score 1, 2 and 3 which correspond to a mitotic cell count ranges of 0-7, 8-14, and >14 per 10 HPFs, respectively), whereas KI was represented as percentage of Ki67-positive cells. To enable us to compare KI and MI on the same scale, we converted the patients’ mitotic scores into mitotic cell counts (in 10 HPFs) and then into percent mitotic cells (as described in the Methods section). Comparing KI values in a grade-wise manner showed a steady increase in mean KI values with Nottingham grade indicating an increasing proportion of cycling cells in higher grade tumors (9.03% to 19.36% to 46.069%) (Fig. 7.4.2B). We then compared the mean MI values grade-wise and found the same trend, though the grade-wise increase in MI was much slower (1.026 to 1.577 to 2.682) (Fig. 7.4.2C).
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Table 1 Clinicopathological characteristics of the breast cancer patient cohort analyzed in this study.

We then analyzed mean KI values for patients in each Mitotic Score category in each grade and found that mean KI values positively correlated with Mitotic Scores in all 3 grades (Fig. 7.4.2A, D). In addition, we observed a steady grade-wise increase in the mean KI value of patients in each Mitotic Score category (Fig. 7.4.2D). These data intriguingly suggested that the relationship between KI and mitotic scores is a dynamic one that varies depending on the tumor’s histological grade.

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<th>Variable</th>
<th>Nottingham Grade I (n = 539)</th>
<th>Nottingham Grade II (n = 638)</th>
<th>Nottingham Grade III (n = 434)</th>
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<td>KI67 % Mean</td>
<td>9.03 (8.25 - 9.81)</td>
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<td>46.06 (43.83 - 48.31)</td>
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<td>MI mean</td>
<td>1.02 (1.01 - 1.03)</td>
<td>1.57 (1.53 - 1.62)</td>
<td>2.40 (2.36 - 2.72)</td>
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<td>Ki67 Adjusted Mitotic Score (KAMS)</td>
<td>0.15 (0.14 - 0.17)</td>
<td>0.13 (0.12 - 0.14)</td>
<td>0.08 (0.074 - 0.08)</td>
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Figure 7.4.2 Analysis of grade-wise relationship between KI and MI.
A. Descriptive statistics of patient samples derived from the Northside Hospital data mining project. Bar plots representing change in mean values across Nottingham Grades, of either B. Ki67 Index (KI) or C. Mitotic Index (MI). D. The box-whisker plots show the change of mean KI based on both a patients mitotic score and Nottingham Grade to depict the dynamic relationship between the KI and mitotic score across Nottingham Grades (ANOVA p < 0.05 for Mitotic score 1 and 2, p = 0.22 for Mitotic score 3). Note the missing bars in mitotic score 1 and 3 which are indicative of the lack of patient samples with those scores in Nottingham Grade III and I, respectively. E. Bar plots representing change in mean KAMS values (ANOVA p < 0.05) with bars indicating standard error.
7.4.3 *The proportion of mitotic cells amongst proliferating tumor cells decreases with increasing tumor grade*

In order to further probe the dynamic relationship between KI and MI, we calculated a MI-to-KI ratio (percent MI-to-percent-KI) for each of the three Mitotic Score categories to represent a new histological metric termed the grade’s KI-adjusted mitotic score (KAMS). In Fig. 7.4.2E, a bar-graphical representation of KAMS values for all three grades, clearly shows that the NGIII patients have a much lower KAMS value than NGI patients (p<0.05 by ANOVA for NGI vs NGII vs NGIII, therefore we can reject the null hypothesis that all three grades have equal means for their MI/KI ratios; post hoc tukey values: for NGI vs NGII: p=0.00273; for NGII vs NGIII: p<0.005; for NGI vs NGIII: p<0.005). Our data uncovers a previously unrecognized fact about high-grade breast carcinomas, viz., that the proportion of mitotic cells amongst proliferating cells is vastly diminished, although the number of Ki67-positive cells is very high. These data are consistent with the idea that early-grade cancers focus heavily on mitotic cell division (they have higher KAMS). The relatively lower KAMS in higher-grade cancers suggest that they are less division-focused and perhaps more dedicated to the interphase activity of metastatic dissemination. These startling results, by being both confirmatory as well as uniquely revealing, unmask several nuances that paint a more fine-grained portrait of proliferation within tumors. While they confirm the idea that both KI and MI increase with advancing grade, they uncover the hitherto unappreciated and almost paradoxical fact that the proportion of mitotic cells amongst the proliferating population (which reflects the mitotic propensity and the kinetics of cell cycling of proliferating cells in the tumor) actually declines with increasing tumor grade.
7.4.4 Rational integration of KI and MI improves stratification of breast cancer patients

Given that our data underscore how the agenda of low-grade tumors is potentially distinct from the agendas of high-grade tumors, we investigated the prognostic value of our new metric KAMS, and evaluated its effectiveness as a classifier for improving patient risk-stratification. Using a threshold value of KAMS for each grade, we stratified patients in each Nottingham grade and performed log-rank tests for their overall survival. Although KAMS was unable to significantly stratify NGI, patients from NGII and NGIII were stratified into subgroups whose survival probabilities were significantly different from each other (p <0.05) (Fig. 7.4.3Ai, ii, iii). Importantly, we found that patients with below-threshold KAMS values in NGII and NGIII, had poorer overall survival (Hazard Ratio (95% CI): NGII=2.898 (1.375-6.107), NGIII = 1.713 (1.035-2.837)), indicating that our metric indeed had a strong risk-predictive value above and beyond that provided by the Nottingham grading variables, particularly in NGII and NGIII where higher KAMS values portend better overall survival.

Having identified that KAMS allows effective stratification of NGII and NGIII into groups with significantly different survival probabilities (Fig. 7.4.3A), we next asked if incorporation of a KAMS-based classification step subsequent to conventional Nottingham classification, would improve stratification of patients in NGII and NGIII to enable their funneling towards more optimal treatment choices. We therefore evaluated a patient grade adjustment model, which creates an adjusted Nottingham classification based on patients’ KAMS values. Alterations in Nottingham Grades was only done on those patients who were in the original NGII or NGIII grades, due to a lack of a clear and significant separation of high/low risk KAMS populations for NGI (See Fig. 7.4.3A). Based on the results shown in Fig. 3A for NGII and NGIII patients, an above-threshold KAMS value was deemed as low-risk and a below-
threshold KAMS value was deemed as high-risk for the purpose of grade adjustment. Low-risk, above-threshold KAMS patients originally designated as NGII were adjusted down to NGI. Higher-risk, below-threshold KAMS patients from NGII were retained in NGII. Low-risk, above-threshold KAMS patients originally assigned to NGIII were adjusted down to NGII. Higher-risk, below-threshold KAMS patients from NGIII were retained in NGIII. Thus the adjusted low-risk group consisted of original NGI patients along with the low-risk subgroup of the original NGII patients. Adjusted NGII patients were composed of KAMS-stratified high-risk patients originally in NGII along with KAMS stratified low-risk subgroup from original NGIII patients. Finally, the adjusted NGIII was made up exclusively of high-risk patients from the original NGIII (See schematic in Fig. 7.4.3B, middle panel for grade adjustment model).

Intriguingly, our grade adjustment model reassigned histological grades of 39% of the patients in our dataset. Fig. 3B depicts Kaplan-Meier survival graphs of patients as graded by the traditional “original” Nottingham grading system compared with the survival graphs of the patients stratified and reclassified employing a KAMS classifier. The adjusted system boasts a wider separation between overall survival curves with adjusted low-risk group (n =774) OS being 95.48%, adjusted moderate-risk group (n =727) OS at 87.62%, and adjusted high-risk group (n =110) having an OS of 78.18%). The striking increase in hazard ratios observed after grade adjustment and the decrease in model fit statistics (where a decrease in \(-2\log L\), AIC and SBC indicate that the adjusted model fits actual outcomes better than the currently used Nottingham Grading System) (Fig. 7.4.3Ci) both testify to the dramatic improvement in patient stratification and more accurate risk-segmentation of patients using our KAMS classifier.

We then determined the concordance index (C-index) values for patient stratification using the original Nottingham Grading System and compared it to the c-index obtained with our
grade adjustment model, based on 100 randomizations of patients into 60% training set and 40% test set (p<0.001 between the two c-indices using t-test). The c-index is a measure of concordance for time-to-event data, in which increasing values between 0.5 and 1.0 indicate improved concordance (beyond what would be predicted by chance alone) between predicted and actual outcomes, and therefore measures true discrimination (value of 1 would imply perfect discrimination). C-index of 0.824 for our KAMS-based grade adjustment model (Fig. 7.4.3Cii) indicates that if this system is used on a randomly selected pair of patients, 82% of the time, a patient who is assigned to a higher (or lower) risk group actually has a worse (or better) prognosis. Importantly, C-index determination clearly demonstrated superiority of our model in comparison to the prognostic information afforded by the traditional Nottingham Grading System (Fig. 7.4.3Cii) demonstrating that the integration of KI and MI yields a new layer of crucial and hitherto overlooked risk-predictive information.

7.4.5 Incorporation of a KAMS classifier improves discrimination of high- and low-risk samples

Analysis of the distribution of various breast cancer subtypes among our patient cohort prior to, during, and after our KAMS-based grade reassignment clearly shows the ability of our metric to distinguish between high- and low-risk breast cancer subtypes. For example, the KAMS classifier designated low-risk subgroup in NGII is more enriched for Luminal A patients (who tend to have good outcomes) compared to the NGII high-risk subgroup (Suppl. Fig. 1). Similarly, the NGIII high-risk subgroup designated by the KAMS classifier is more enriched for triple-negative patients (who generally have poorer outcomes) compared to the NGIII low-risk group (Suppl. Fig. 1). Particularly impressive is also the ability of the KAMS-classifier to segregate patient sub-groups based on their KI values which provide a good measure of the
aggressiveness inherent in the tumor samples. For example, high-risk subgroups in NGII and NGIII tend to have higher mean KI values (25.65 % for NGII, 76.15% for NGIII, respectively) compared to the corresponding low-risk subgroups (8.56% for NGII and 35.86% for NGIII, respectively) (Suppl. Fig. 2). Particularly striking are the sharp KI cutoffs apparent in the adjusted risk groups after KAMS-based patient re-stratification. For instance, 100% of NGII patients adjusted into the low-risk subgroup have KI values below 24% and 99.1% of patients in the adjusted high-risk group have KI values above 48% (Suppl. Fig. 2). In sum, our novel histological metric (KAMS), which rationally integrates KI and MI, yields a new layer of information that more completely harnesses the risk-predictive values of both KI and MI, and strengthens the prognostic accuracy of tumor histological grade.

Figure 7.4.3 Incorporation of KAMS classifier improves patient stratification
Ai, ii, iii. Kaplan-Meier survival plots showing stratification of patients with NGI (KAMS threshold = 0.154), NGII (KAMS threshold = 0.1167) or NGIII (KAMS threshold = 0.0395) based on a KAMS threshold of either the average KAMS (NGI and NGII) or an ideal threshold (NGIII). B. Kaplan-Meier survival plot of patients stratified by the original Nottingham grading system (left panel), who were re-stratified as shown in the schematic representation (middle panel) using the KAMS classifier, thus yielding the Kaplan-Meier survival curves of patients after the grade adjustment (right panel). Overall survival for Grade I, II and III changed from 95.36, 90.75, 85.02 to 95.48, 87.62, 78.18 days, respectively. Ci shows the decrease of all 3 model fit statistics for the adjusted model alongside a marked increase in hazard ratios (using Grade 1 as the reference point for both models) both indicating the superior fit of the adjusted model (even though both all HR’s were significant). Significance for Kaplan-Meier was determined with the log-rank test while for hazard ratios it was via chi-square. Cii. Bar plot showing comparison of mean C-Index of 100 bootstraps of our dataset (using 60% cases as testing set and 40% cases as validation set), between the original Nottingham Grading and KAMS adjusted system (p<.001).
Suppl. Fig. 1. Schematic representing distribution of tumor subtypes before, during and after the re-stratification of original Nottingham Grades using KAMS classifier.
Suppl. Fig. 2. Schematic depicting distribution of KI values before, during and after the KAMS-based re-stratification of patients from original Nottingham Grades.

7.4.6 *Extraction and integration of KI and MI from the same microscopic field enhances patient risk stratification*

Our afore-stated data showed that the rational integration of KI and MI vastly improves patient segmentation into risk groups. KAMS, however, integrates KI and MI data from non-overlapping fields as KI and MI are currently determined from different slides in diagnostic pathology. The determination of KAMS also involves certain approximations particularly related
to cellularity. However, the compelling results obtained with KAMS-based stratification motivated us to examine whether the extraction of KI and MI from the same field, and the derivation of the ratio of mitotic cells to the proliferating cells (i.e., the M-to-P Ratio) in that field would similarly improve patient stratification. To this end, we optimized a first-of-its-kind 4-color immunofluorescence protocol and stained 233 archival paraffin-embedded NGI, NGII and NGIII breast carcinoma tumor tissue slides [in which tumor areas had been marked out by referring to the corresponding pre-marked H&E-stained slide (obtained from Northside Hospital, Atlanta)] with antibodies against (i) Ki-67, (ii) p-H3- a marker for mitosis- and (iii) α-tubulin in order to visualize microtubules, and stained DNA using TOPRO. Using confocal immunofluorescence microscopy, Ki-67-positive cells and mitotic cells were counted simultaneously in the same 10 randomly-selected HPFs (at least 500 nuclei were counted for each sample) and the M-to-P ratio was derived for each sample by calculating the ratio of number of p-H3-positive cells to number of Ki67-positive cells in the same 10 HPFs. We observed that different fields showed different numbers of mitotic cells (readily distinguished via high-contrast p-H3 staining) and Ki67-positive cells (Fig. 7.4.4A, top and bottom rows). Visualization of microtubules allowed accurate determination of the precise phase of mitosis observed in the tumor cells. We found a striking grade-wise increase in the mean KI values of the samples (35.89% in NGI to 51.77% in NGII to 78.05% in NGIII) with all differences being statistically significant (Fig. 7.4.4B). We observed a similar statistically significant grade-wise trend with MI also with mean MI values climbing from 6.80% in NGI to 9.04% in NGII to 11.51% in NGIII (Fig. 7.4.4C). Most importantly, we found a clear grade-wise decrease in the M-to-P ratio (Fig. 7.4.4D). These trends are all consistent with what had been observed with KAMS in the context of the large dataset, indicating that the proportion of mitotic cells among
Ki67-positive proliferating cells indeed declines as we go from NGI to NGIII. In sum, our data show that although the numbers of mitotic cells and proliferating cells increase in a grade-wise manner, the fraction of dividing cells amongst the proliferating cell population actually decreases with advancing tumor grade.

Figure 7.4.4 Differential fields depicting mitotic propensities observed in two breast tumors.
The tumors are immunostained for Ki67 (blue), p-H3 (green), microtubules (grey) and DNA (red). Sample in top row has 13 Ki67-positive cells, 1 p-H3-positive cells in a field, M-to-P ratio for field 1 =1/13 X 100=7.69. Sample in bottom row has 13 Ki67-positive cells, 2 p-H3-positive cells, M-to-P ratio for field 2 =2/13X 100=15.3. Bar plots representing change in mean values across Nottingham Grades, of either B. % Ki67 (ANOVA, p=0.05), C. % mitotis (ANOVA, p<0.0001) or D. M-to-P ratio (ANOVA, p<0.0001).
7.4.7 Incorporation of an M-to-P ratio classifier can enhance patient stratification provided by both the Nottingham Grading System and Ki67 groups

After establishing that our 4-color immunofluorescence microscopy of tissue samples yielded grade-wise trends in KI, MI and M-to-P ratios that paralleled those observed with KAMS, we evaluated whether incorporation of the M-to-P ratio classifier could augment and improve the risk-stratification provided by (i) Nottingham Grading System, and (ii) the KI of patients (using the Ki-67 labeling index cutoffs adopted by the St. Gallen International Expert Consensus to offer therapeutic decisions). Our Nottingham grade and KI group adjustment model (Fig. 7.4.5A) utilized the general principle that high-risk subgroups from NGI and NGII (or KI Group I and KI Group II) would move into the next higher-risk Nottingham Grade or KI Group. In addition, the low-risk subgroup from NGIII (or KI Group III) was moved down into the “adjusted” moderate-risk group. Assignments of patients in each Nottingham Grade or KI group into high- or low-risk subgroups, and the rationale for the grade reassignments (depicted in Fig. 7.4.5A) were based on the following data: (i) Stratification of NGI by the M-to-P ratio classifier showed that patients with a higher M-to-P ratio had poorer overall survival (Fig. 7.4.5Bi), indicating increased risk; (ii) stratification of NGII and NGIII individually (data not shown) or a combined NGII + NGIII patient cohort by the M-to-P ratio classifier showed that patients with a higher M-to-P ratio had better overall survival (Fig. 7.4.5Bii), indicating decreased risk. Nottingham grade reassignment resulted in the reassignment of the tumor grades of 35% of patients into “adjusted” groups showing comparable or higher hazard ratios (HRs) compared to the original groups (HR adjusted NGII =1.168(0.466-2.929) versus original 1.24(0.599-2.522); HR for NGIII=1.911(0.849-4.305) versus original 1.33(0.645-2.743)) and a striking improvement in model fit statistics. Notably, the classifier did enrich the NGIII high-risk
group with triple negative patients (Suppl. Fig. 3), which is consistent with the aggressive phenotypes and poor outcomes associated with triple negative breast cancer. The M-to-P classifier also enriched the high-risk subgroups in NGII and NGIII with patients bearing higher mean KIs (mean KI values: NGII high-risk subgroup=22.2%, NGIII high-risk subgroup=33.6%) compared to the low-risk subgroups (mean KI values: NGII low-risk subgroup=12.8%, NGIII low-risk subgroups=19.8%) in these grades (Suppl. Fig. 4). These data demonstrate that extraction of KI and MI from the same field, and incorporation of the M-to-P ratio thus derived into our grade adjustment model, uncovers the risk contribution of a fundamental aspect of tumor biology (viz., the tumor’s mitotic propensity) and significantly improves patient stratification compared to the Nottingham grading system.

We then reclassified patients originally grouped according to their KI values (KI Group I: KI <15%, KI Group II: 15%<KI≤30%, KI Group III: KI >30%); this allowed us to assess whether the incorporation of our M-to-P ratio classifier could add to the prognostic value of KI, a biomarker with strong prognostic value. Interestingly, stratification of KI Group I by the M-to-P ratio classifier showed that patients with a higher M-to-P ratio had poorer overall survival (Fig. 7.4.5Ci), indicating increased risk. Similarly, stratification of KI Group II (Fig. 7.4.5Cii) and KI Group III (Fig. 7.4.5Ciii) demonstrated that patients with a higher M-to-P ratio had better overall survival, indicating decreased risk. These trends strikingly mirrored the trends observed with the Nottingham Grades I, II and III (Fig. 7.4.5Bi and 7.4.5Bii). Utilizing our KI Group adjustment model (Fig. 7.4.5A), 53.3% of patients were assigned to “adjusted” risk groups that were different from the ones they originally belonged to. KI Group adjustment led to a dramatic improvement in patient stratification as evidenced by the fact that the Kaplan-Meier survival graphs of patients in adjusted groups show statistically significant stratification (compare
survival graphs of patients in the original KI groups (Fig. 7.4.5Civ) versus adjusted KI Groups (Fig. 7.4.5Cv)). The marked decrease in the 12logL, AIC and SBC values also testify to the vastly improved model fit observed upon KI group reassignment via the M-to-P ratio classifier (Fig. 7.4.5Cvi). Importantly, adjustment of Nottingham grades and KI groups via incorporation of an M-to-P ratio classifier, resulted in significant improvements in concordance indices for the adjusted risk groups (Fig. 7.4.6A), implying a superior patient classification system that segments patients into risk groups more accurately. We also observed that the M-to-P classifier enriches (i) the low-risk subgroup in KI Group I for Luminal A patients, and (ii) the high-risk subgroup in KI Group III for triple negative cancers (Suppl. Fig. 5). In sum, our data demonstrate that integration of KI and MI from the same field to profile the M-to-P ratio of tumors yields a new level of risk-predictive information and significantly enhances the prognostic accuracy of tumor grade and KI alone.
Figure 7.4.5 Schematic illustrating the re-stratification/adjustment of patients using M-to-P ratio classifier.

A. for patients originally graded using either the original Nottingham Grading system or Ki67 grading set by the St-Gallen Consensus Conference. Bi shows stratification of NGI patients using M-to-P ratio (threshold = 30.952) with the above threshold group having lower survival (82.93 vs. 66.67 days) than the below threshold group. Bii shows the stratification of the poorly differentiated cases, NGII and NGIII, with M-to-P ratio (threshold = 23.404) with the below threshold group having lower survival (70.73 vs 83.33 days) than the above threshold group. Biii shows the reduction of fit statistics when using the adjusted M-to-P based model versus the original model based on Nottingham Grading System. Ci shows M-to-P based survival stratification of KI group I using M-to-P ratio (threshold = 22.222) with above threshold group...
having lower survival (71.05 vs 88.57 days) than the below threshold group. Cii shows M-to-P based survival stratification of KI group II (threshold = 21.4286) with above threshold group having higher survival (60 vs 82.35 days) than the below threshold group. Ciii shows M-to-P based survival stratification of KI Group III (threshold = 13.3333) with above threshold group having higher survival (66.67 vs 87.8 days) than below threshold group. Civ,v show the Kaplan Meier curves based on the original and adjusted KI grading respectively, depicting a widening of overall survival stratification using the adjusted model for KI group I, II and III from 79.45 69.05 77.5 to 88.57 79.38 65.08 days, respectively. Cvi shows the model fit statistics for the original KI model versus the adjusted model based on M-to-P ratio. p-values shown for the respective Kaplan-Meier curves were based on the log-rank test.
Suppl. Fig. 3. Schematic representing distribution of tumor subtypes before, during and after the re-stratification of patients from original Nottingham Grades using the M-to-P ratio classifier.
Suppl. Fig. 4. Schematic depicting distribution of KI values before, during and after the re-stratification of patients from original Nottingham Grades using the M-to-P ratio classifier.
Suppl. Fig. 5. Schematic depicting distribution of tumor subtypes before, during and after the re-stratification of patients from original KI-based Groups using M-to-P ratio classifier.
7.4.8 M-to-P profiling of tumors uncovers fundamental differences in their proliferation landscapes

The M-to P ratio of a tumor sample illuminates the proportion of proliferative cells that are actively undergoing cell division; in doing so, this metric describes the cell cycling kinetics within the proliferative population. In other words, a higher M-to-P ratio is indicative of faster cell cycling kinetics within the proliferative population compared to a tumor with a lower M-to P ratio. Since the duration of mitosis is the least variable phase of the cell cycle, we wanted to examine the grade-wise difference in the duration of interphase in the proliferative cell population. Using the principle that the number of labeled cells in each phase of the cell cycle is correlated with the duration spent in that particular phase of the cell cycle, we were able to compute the duration of interphase relative to the duration of mitosis. We found that the average duration of interphase in the proliferative cells of an NGII tumor is 1.062 times the duration of interphase in an NGI tumor. Similarly, the duration of interphase in an NGIII tumor was 1.435 times the duration of interphase in an NGI tumor (Fig. 7.4.6B). M-to-P ratio profiling thus revealed that on average, proliferating cells in higher-grade tumors spend a much longer duration in interphase compared to proliferating cells in low-grade tumors.

Figure 7.4.6 Comparison between the mean C-index and the adjusted KI.
A. shows the comparison between the mean C-index of 100 bootstrapped samples stratified using models based on either the original and the adjusted KI grading or the original and the adjusted Nottingham Grading system (t-test p < .0001 between each model). The adjusted models clearly showed an improved discriminatory ability with the adjusted KI grading having the overall best concordance. B. Schematic representation of the duration of interphase associated with NGI, II and III; relative to NGI, assuming that the duration of mitosis stays constant. The following formula was used to determine interphase time using the M-to-P ratio:

\[
\frac{M - to - P\; ratio}{\#\; cells\; labelled\; with\; p-H3\; \#\; cells\; labelled\; with\; Ki67} = \frac{1\; hour\; mitosis}{1\; hour\; mitosis + X\; hours\; in\; interphase+1}
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7.5 Discussion

Prognostic biomarkers such as KI and MI have been widely adopted by clinicians in breast cancer diagnostic pathology to more accurately estimate risk and ascertain the most optimal treatment path. Historically, MI has demonstrated strong prognostic value evident even in prospective studies. As a result, MI is used as the proliferation marker within the NGS. Despite the prognostic merits of KI being well-documented, KI has never been integrated into the NGS and has remained a widely-determined, independent prognostic marker used in decision-making on adjuvant treatment strategies. Although many pathologists report KI in addition to the traditional NGS parameters, the existing guidelines of the ASCO do not require KI to be routinely determined for all samples. Importantly, both the NGS and KI still have limited prognostic accuracy, thus emphasizing that better risk estimation measures that can improve patient stratification are direly needed.

We believe that the independent determination of KI and MI and their separate/disjointed consideration in decision making (which disregards the fact that mitosis is an integral part of the proliferative cell cycle), in essence splinters their utility and fails to harness the full prognostic potential of these indices. We postulated and verified that the prognostic accuracy of MI was also compromised by subjectivity and errors in its visual determination. We first showed that visual recognition of mitotic cells for tumor grading consistently underestimated the number of mitotic
cells thereby potentially compromising the accuracy of the histological grade assigned to samples (Fig. 7.4.1Ci). Furthermore, we convincingly demonstrated that p-H3 is a far more reliable and readily discernable marker for mitosis (Fig. 7.4.1B and 7.4.1Cii) owing to its clear and contrast-rich distribution pattern. Our observations corroborate earlier findings and emphasize that the use of p-H3 as a marker for mitosis reduces inter-observer variability, enhances reproducibility and accuracy of MI determination.

We then evaluated the relationship between KI and MI in a retrospective analysis of over 1600 patient records. We first brought KI and MI on the same measurement scale by defining a new metric (KAMS) wherein we normalized the mitotic scores of samples using the independently-derived KI values of the respective samples. We found that although KI and MI both increase with tumor grade, the grade-wise increase in MI is much lower than that in KI (Fig. 7.4.2B, C). More importantly, the relationship between KI and MI varies dynamically depending on the Nottingham grade (Fig. 7.4.2D). This broken relationship between KI and MI hints that mitosis may not always constitute the same proportion of the proliferative cell cycle.

After establishing p-H3 as a superior marker for mitotic cells, we rationally married KI and MI and brought them on the same scale of measurement by extracting both KI and MI (using p-H3 as the mitotic marker) from the same microscopic field to derive the M-to-P ratio for 233 tumor samples. Despite the decades-long debates raging about the relative merits of KI and MI, and the degree of correlation between these two prognostic biomarkers in different cancer types, no one disputes the notion that faster tumor growth is a sign of more aggressive disease that poses greater risk to the patient. Faster tumor growth can result from two possible scenarios: (i) KI increases and MI increases proportionally with KI, or (ii), both KI and MI increase but the increase in MI is not proportional to the increase in KI. Our data (Fig. 7.4.4B, C) show the latter
possibility to be true. Moreover, recent studies have clearly divulged that the majority of cells within the proliferative cell population in a tumor, are not actually dividing (i.e., are not in M-phase of the cell cycle) but are instead, populating interphase. The more speedily cells transit through the cell cycle (in other words, the less they linger in interphase), the higher will be the proportion of mitotic cells observed in the proliferating population. In other words, the proportion of mitotic cells amongst proliferating cells (M-to-P Ratio) provides a measure of the kinetics of cell cycling, or the mitotic propensity of the proliferative population; this new metric provides a good measure of the risk posed by the proliferative population due to erroneous mitoses that could drive chromosomal instability and intratumoral heterogeneity. While neither the idea of replacing MI in the NGS with KI, nor enhancing survival analysis by independently observing KI in addition to MI are novel, our hypothesis-driven approach of rationally deriving a new risk-predictive metric (M-to-P ratio) by integrating both MI and KI from the same microscopic field, is truly unprecedented and innovative. Importantly, our analysis of the M-to-P data revealed the hitherto unappreciated fact that the proportion of dividing cells within the proliferative cell population declines with increasing tumor grade (Fig. 7.4.4D). Our findings indeed reveal a fundamental aspect of tumor biology, viz., that cells in low-grade tumors undergo frequent mitoses whereas cells in high-grade tumors, undergo mitoses less frequently and spend longer durations of time languishing in interphase (Fig. 7.4.6).

The use of our M-to-P ratio classifier to stratify the Nottingham grades and the KI-based groups into high- and low-risk subgroups also revealed other startling findings with deep clinical implications. For NGI and KI Group I, patients with high M-to-P Ratios had poorer outcomes compared to patients with lower M-to-P ratios. This suggests that in low-grade tumors, a high mitotic propensity predisposes the tumor to generate abundant clonal diversity that could result
in the rapid evolution of more aggressive clones and in decreased OS. A reversal in this trend is apparent with NGII and NGIII, and KI Groups II and III where lower M-to-P ratios are associated with poorer outcomes. These data suggest that for high-grade tumors, low M-to-P ratios indicate a switch in the tumor’s agenda to a program less focused on mitosis and perhaps more focused on migration and metastatic dissemination, resulting in worse clinical outcomes. We envisage that the inflection point in the clinical implications of M-to-P ratios embodies a fundamental switch in the tumor’s agenda from division to dissemination, and a fundamental reprogramming of cellular states accompanying this phenotypic switch. Thus, by revealing finer grade-wise details about the mitotic and proliferation landscape of tumors, the M-to-P ratio incorporates the hidden agendas of evolving tumors into the 4-dimensional movie of cancer progression, rather than giving us static snapshots of the disease as MI and KI did.

The use of M-to-P ratio in routine diagnostic pathology would require the development of a clinically facile 2-color immunohistochemistry method that would not only vastly improve the accuracy of patient risk-stratification but more importantly, would involve no addition to a pathologist’s current workload. Future work would also require the development of an ideal model for integrating M-to-P Ratio into the breast tumor grading system in order to enhance the prognostic accuracy of histological grade. Further research involving the M-to-P ratio profiling of large cohorts of breast tumors could also address the question of whether fundamental differences in this metric could illuminate inherent differences in tumor biology between the different subtypes of breast cancers and reveal insights about the susceptibilities of tumors to chemotherapeutics and anti-mitotic treatment approaches.
7.6 References


8. CONCLUSIONS

An emerging yet nascent paradigm in the field of cancer biology is ITH, lending the view that tumors are a mélange of remarkably diverse (both genotypically and phenotypically) and distinct cells. The degree and extent of this expansive clonal heterogeneity has largely gone neglected in the light of the long-held belief that tumors are comprised of genetically-identical cells harboring superlative karyotypic composition. No wonder, there have been no studies yet which have been able to offer a rapid, facile, cost-effective and clinically-adaptable read-out of ITH.

In this collection of studies, we have presented several pioneering, well-founded contributors to tumor aggressiveness that can not only serve as reliable risk predictors of breast cancer metastasis and patient survival but may also trend-line with ITH. Rational-thinking and logical reasoning predict that enhanced rate of erroneous mitosis in chromosomally-unstable cells bearing extra centrosomes would drive emergence of clonal diversity. Our statistically-validated algorithm, that defines a measurable index called Centrosome Amplification Score (CAS) accounting for both numeral (CASi) and structural (CASm) amplification, offers a comprehensive and a quantitatively precise measure of aberrant centrosomal status in breast tumors. Additionally, our novel metric that rationally integrates KI and MI by defining a combined “M-to-P ratio” which serves as a better clinical prognosticator and predictor of metastatic risk associated with an early-stage tumor, than either KI or MI alone. Both these innovative metrics, CAS and M-to-P ratio, may offer groundbreaking insights into a tumor’s evolutionary agenda and can be integrated into current diagnostic practices to improve clinical decision making.
Since centrosome amplification and clustering are cancer cell-specific phenomena, centrosome-clustering pathways may also serve as attractive theranostic targets. The non-essential role of HSET in normal somatic cells and its crucial requirement for the viability of cancer cells, together paint a picture of HSET as a seemingly ideal cancer-selective drug target that is pending validation in animal studies. This proposition is bolstered by the observation that HSET depletion using RNAi induces robust spindle multipolarity and subsequent apoptosis in aggressive breast cancer cells and prostate cancer cells. Unquestionable dependence of cancer cells on HSET function and the association of HSET overexpression with the aggressiveness of certain cancers offer a strong rationale for the development of HSET as a risk-predictive biomarker. Given that HSET serves as a panacea for cancer cells to rescue them from a catastrophic multipolar mitosis, it is an important teamplayer in their agenda for relentless survival via the process of intratumoral heterogeneity. In the light of HSET’s myriad clinical implications in driving tumor heterogeneity and disease progression via clustering-dependent as well as independent pathways, HSET depletion can also potentially open up manifold therapeutic avenues for single agent or combination therapies, which is not limited to tumors harboring extensive centrosome amplification. As of today, there are two commercially available HSET inhibitors (AZ82 and CW069) whose effectiveness in animal models and in the clinic are yet to be demonstrated. From a therapeutic standpoint, it is important that studies scrutinize the entire spectrum of repercussions following the pharmacological manipulation of a crucial cytoskeletal motor such as HSET. Thus, there is a compelling need to launch a systematic search for putative HSET inhibitors for selective tumor targeting and exploit HSET’s potential in enabling risk-based patient stratification, thus ensuring a more focused chemotherapeutic approach as we step into the era of personalized medicine.
The emergence of tumor cells with genetically divergent cell populations is pertinent for the cell to gain intratumor clonal heterogeneity. By employing statistical analysis on a large clinical dataset, we showed that early-stage tumors which undergo higher “mitotic turnover”, allow for promoting generation of genetic diversity and may pose a higher risk compared to those which are not mitotically active during their early stages. Our rationale is that the rate of mitosis (mitotic frequency or mitotic turnover) is a “means” to produce chromosomal missegregation which equates with chromosomal instability. Thus, given the fact that mitotic divisions in cancer cells are error-prone due to compromised checkpoints, these strategies set grounds for the emergence of clonal heterogeneity within the same tumor, which prototypes a tumor with high mitotic activity as the one with poor prognosis, and high metastatic risk. However, the mitotic agenda gives way to the migratory agenda when tumors are highly heterogenous as migration facilitates the metastastic journey. Thus, for low grade tumors (well differentiated, low Ki67 scores), it is likely that a higher proportion of Ki67-positive cells be in mitosis as compared to high grade (poorly differentiated, high Ki67 scores) where the focus is less on generating diverse phenotypes and more on dissemination/metastases (or “breaking-free”). We postulate that once clonal heterogeneity is maximized, cancer cells move on from their “mitotic cell division heavy” agenda and focus on their migratory program which is a precursor of distant metastases. This notion is in line with the theory of genomic convergence which suggests that while early stage tumors possess high ITH, later stage tumors are relatively homogeneous.

Thus together, CA and frequent mitosis at an early stage allow cancer cells to rapidly generate a population of genetically diverse subclones, thus providing a relatively varied genetic reservoir. Additionally, through error-prone mitoses, chromosomal instability would continually
expand the phenotypic pool within the cancer cell population ensuring that the cells have the appropriate arsenal to be able to adapt to the dynamic environment including interaction with the continuously changing microenvironment, varying oxygenation levels due to insufficient vasculature and eventually chemotherapeutic encounters. It is highly plausible that the distinct CA causing mutations coupled with the mutations resulting in deregulation of cellular proliferation, shape the early events in tumorigenesis.

We thus conceptualize that CA acts as the “driver” and mitosis as the “vehicle” of ITH. This essentially alludes to the fact that the presence of amplified centrosomes in cancers may proxy the extent of ITH but “that alone” cannot generate ITH unless mitosis takes place. Subsequent to an erroneous mitosis, chromosomes missegregate yielding CIN, aneuploidy and eventual generation of ITH over several rounds of division. Therefore, CAS, HSET expression and M-to-P ratio are undeniably the integral measures of ITH and consolidation of these three factors may yield a reliable and comprehensive “ITH index” which can be brought to the clinic quicker than other whole genome based sequencing modalities.

Next-generation sequencing has sufficiently revealed the existence of ITH in a wide variety of tumors and its significance in determining patient outcomes. Several studies have not only determined correlations between ITH and clinical tumor progression, but also revealed that chemotherapeutic interventions acted as a catalyst for clonal evolution. However, NGS is undoubtedly labor intensive and expensive, requiring specialized expertise in handling technical as well as analytical aspects. In addition, analyzing the dominant clones within the tumor population may result in masking critical aspects of these genetically diverse cell populations, thus resulting in the underestimation of risk. As a result, targeted therapies would hardly be effective at conquering the war against cancer. In addition, targeting one type of clone may
inadvertently modify the tumor landscape leading the selection pressure to allow the domination of distinct clones.

The dynamically evolving tumor agenda commands the urgent need to develop novel and more comprehensive biomarkers and integrative therapeutic strategies. An effective strategy would be to explore compliant therapeutic targets that may limit the extent of ITH at an early stage, thus rendering it relatively manageable.